Neurokinin-1 Receptor Antagonists Protect Mice from CD95- and Tumor Necrosis Factor-α-Mediated Apoptotic Liver Damage

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ABSTRACT

Previously, we have shown that primary afferent neurons are necessary for disease activity in immune-mediated liver injury in mice. These nerve fibers are detectable by substance P (SP) immunocytochemistry in the portal tract of rodent liver. Antagonists of the neurokinin-1 receptor (NK-1R), which is the prime receptor of SP, prevented liver damage by suppressing the synthesis of proinflammatory cytokines. Here, we investigated the influence of primary afferent nerve fibers, SP, and NK-1 receptor antagonists on hepatocyte apoptosis in vivo induced by administration of anti-CD95 monoclonal antibody (mAb) to mice. Depletion of primary afferent nerve fibers by neonatal capsaicin treatment prevented CD95-mediated activation of caspase-3, measured as enzymatic activity in liver homogenates or by demonstration of hepatocellular immunoreactivity for active caspase-3 in liver slices, and liver damage. This effect was reversed by administration of SP to anti-CD95 mAb-treated mice depleted from primary afferent neurons. The presence of the NK-1R on mouse hepatocytes was demonstrated by immunocytochemistry and flow cytometry. Intraperitoneal pretreatment with the NK-1 receptor antagonists (2S,3S)-cis-2-(diphenylmethyl)-N-[2-methoxyphenyl]-methyl)-1-azabicyclo(2.2.2.)-octan-3-amine (CP-96,345) or (2S,3S)-3-(3,5-bis(trifluoromethyl)phenyl) methoxy)-2-phenylpiperadine (L-733,060) dose dependently protected mice from CD95-mediated liver injury. Similar results were obtained when apoptotic liver damage was induced by administration of tumor necrosis factor-α to α-galactosamine-sensitized mice. In conclusion, SP, probably by binding to its receptor on hepatocytes, might aggravate apoptotic signals in these cells. Because NK-1 receptor antagonists not only suppress the proinflammatory cytokine response in the liver but also prevent liver cell apoptosis in vivo, they might be suitable drugs for treatment of immune-mediated liver disease.

Capsaicin-sensitive primary afferent neurons, mainly equipped with unmyelinated C-fibers, are well known to transmit nociceptive afferent signals to the spinal cord in response to tissue injury and inflammation (Holzer, 1988). Moreover, they mediate neurogenic inflammation in peripheral organs such as respiratory and gastrointestinal tract and skin (Holzer, 1988). The proinflammatory effects are most likely mediated by local effector functions of these neurons, i.e., by local release of neuropeptides such as tachykinins and calcitonin gene-related peptide from their axon terminals (Holzer, 1988). Accordingly, increased quantities of these neuropeptides have been detected in inflamed kidneys of mice suffering from a systemic autoimmune disease (Bracci-Laudiero et al., 1998). The tachykinin substance P (SP) is considered as a serious candidate mediator of the local effector function of primary afferent neurons (Harrison and Geppetti, 2001). In vitro, SP elicits activation of the proinflammatory transcription factor nuclear factor-κB (Marriott et al., 2000) and activates immune cells to produce cytokines (Lotz et al., 1988; Rameshwar et al., 1994).

SP preferentially binds to the G protein-coupled neurokinin-1 receptor (NK-1R; Harrison and Geppetti, 2001). Studies using either NK-1R antagonists or mice genetically deficient for the NK-1R have indicated a major role for this receptor in asthma and chronic bronchitis, intestinal inflammation, pancreatitis, arthritis, and resistance to infection (Kincy-Cain and Bost, 1996; Quartara and Maggi, 1998; Harrison and Geppetti, 2001).

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ABBREVIATIONS: SP, substance P; NK-1R, neurokinin-1 receptor; SEB, Staphylococcus enterotoxin B; LPS, lipopolysaccharide; CHAPS, (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate); GaN, d-galactosamine; TNFα, tumor necrosis factor-α; TNFR1, tumor necrosis factor-α receptor 1; mAb, monoclonal antibody; PBS, phosphate-buffered saline; CP-96,345, (2S,3S)-cis-2-(diphenylmethyl)-N-[2-methoxyphenyl]-methyl)-1-azabicyclo(2.2.2.)-octan-3-amine; L-733,060, (2S,3S)-3-(3,5-bis(trifluoromethyl)phenyl)methoxy)-2-phenylpiperadine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BSA, bovine serum albumin.
Until now, only limited data are available regarding the role of primary afferent neurons in the liver. These nerve fibers are detectable by SP and calcitonin gene-related peptide immunoreactivity in the portal tract of human (Stoyanova and Gulubova, 1998) and rodent (Markus et al., 1998; Tiegs et al., 1999) livers. Immuno-reactive nerve fibers were capsaicin-sensitive, i.e., they were completely absent in adult mice that have been treated with capsaicin 3 days after birth to permanently deplete the C-fibers (Tiegs et al., 1999). Evidence for a functional role of these capsaicin-sensitive nerve fibers in liver injury has been demonstrated in experimental liver fibrosis induced by common bile duct ligation (Casini et al., 1990). We have shown recently that these nerve fibers are absolutely required for disease activity in several models of immune-mediated liver damage inducible by the lectin concanavalin A in nonsensitized or by the bacterial products Staphylococcus enterotoxin B (SEB) or lipopolysaccharide (LPS) in d-galactosamine (GalN)-sensitized mice (Tiegs et al., 1999; Bang et al., 2003). Accordingly, NK-1 antagonists prevented inflammatory liver disease by inhibiting nuclear factor-κB activation, by suppressing the synthesis of proinflammatory and concomitantly increasing the production of hepatoprotective cytokines (Bang et al., 2003). The tumor necrosis factor-α (TNFa)/TNF receptor-1 (TNFR1) as well as the CD95L/CD95 system become activated during the immune-response elicited by either concanavalin A or SEB or LPS and have been shown to act as downstream signals, inducing hepatocellular apoptosis, which finally results in liver damage (Seino et al., 1997; Schumann and Tiegs, 1999). To shortcut the system, i.e., to directly activate the death receptors TNFR1 or CD95 in vivo, administration of recombinant TNFa to GalN-sensitized mice or of activating anti-CD95 monoclonal antibody (mAb) to nonsensitized mice can be used to induce hepatocyte apoptosis, secondary necrosis and liver damage (Ogasawara et al., 1993; Schumann and Tiegs, 1999).

Because SP has been reported to act as a potential apoptotic peptide in a cell free system (del Rio et al., 2001) and to be involved in kainate-induced apoptosis of hippocampal neurons in vivo (Liu et al., 1999), we wondered whether capsaicin-sensitive primary afferent neurons, their transmitter peptide SP as well as the NK-1 are directly involved in apoptotic liver damage induced by death receptor activation in vivo. Because the NK-1 antagonists CP-96,345 (Snider et al., 1991) and L-733,060 (Rupniak et al., 1996) not only suppressed the inflammatory response in the liver (Bang et al., 2003) but also prevented CD95- and TNFR1-mediated hepatocellular apoptosis in vivo (this study), these drugs might be highly recommended for treatment of immune-mediated human liver disease such as autoimmune hepatitis.

Materials and Methods

Animals. BALB/c mice (age, 6–8 weeks; weight range, 18–25 g) were obtained from the animal facilities of the Institute of Experimental and Clinical Pharmacology and Toxicology of the University of Erlangen-Nuremberg (Erlangen, Germany). All mice received humane care according to the guidelines of the National Institutes of Health, as well as to the legal requirements in Germany. They were maintained under controlled conditions (22°C, 55% humidity, and 12-h day/night rhythm) and fed a standard laboratory chow.

Dosages and Application Routes. For capsaicin pretreatment, newborn BALB/c mice were injected with 100 mg/kg capsaicin s.c. in a volume of 40 μl on the 3rd day after birth. Capsaicin (Tocris/Biotrend GmbH, Köln, Germany) was initially dissolved in 100% ethanol and further diluted with sterile PBS and Tween 80 to a final concentration of 5 mg/ml in 10% Tween and 10% ethanol. Control animals received the solvent without capsaicin. Seven weeks after injection, the effectiveness of the capsaicin pretreatment was assessed by the eye-wiping test by application of 0.1 mg/ml solution of capsaicin (in saline) onto the eye. All control animals wiped the eye rigorously (>30 wipes in 30 s) with a latency of <1 s. Capsaicin-pretreated animals showed a latency of >5 s to the first wipe and did not carry out more than five wipes in 30 s. Most capsaicin-pretreated animals did not respond at all.

All other reagents were injected in a total volume of 250 μl per 25-g mouse. Activating anti-mouse CD95 mAb (Jo2; BD Pharmingen, Heidelberg; Germany) was administered intravenously in pyrogen-free saline at a dose of 120 μg/kg. SP (5 mg/kg, dissolved in pyrogen-free saline; Tocris/Biotrend Chemikalien GmbH, Köln, Germany) was injected intraperitoneally immediately after Jo2 application. Recombinant murine TNFα was purchased from Innogenetics (Gent, Belgium) and administered i.v. at a dose of 6.5 μg/kg 15 min after intraperitoneal administration of 700 mg/kg GalN (Carl Roth GmbH, Karlsruhe, Germany) in pyrogen-free saline.

The NK-1 antagonists CP-96,345 (generous gift of Dr. H. Berg-hof, Pfizer GmbH, Karlsruhe, Germany) or L-733,060 (Tocris/Biotrend Chemikalien GmbH) were administered in doses of 1.25 to 10 mg/kg i.p. 30 min before challenge with anti-CD95 mAb or GalN/TNFα.

Analysis of Liver Enzymes. Hepatocyte damage was assessed 8 h after anti-CD95 mAb or GalN/TNFα administration by measuring plasma enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using an automated procedure (Bergmeyer, 1984).

Determination of Caspase-3 Activity. To determine the activation of caspase-3 in liver tissue of mice, liver homogenates [50% (w/w)] were prepared in lysis buffer containing 10 mM HEPES, pH 7.4, 1 mM 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), and 1 mM diithiothreitol and analyzed using the colorimetric caspase-3 assay kit (Sigma Chemical, Taufkirchen, Germany) according to the manufacturer’s instructions.

Immunocytochemistry of Caspase-3 in Liver Sections. For immunohistochemistry on cryostat sections, liver samples were embedded with OSV 1 tissue-embedding medium (Slee Technik GmbH, Mainz, Germany), frozen in 2-methylbutane (Carl Roth GmbH), and stored at −80°C until use. Cryostat sections of 10 μm were thawed on glass slides, air-dried, fixed for 10 min at 4°C in acetone/methanol (1:1), and used immediately or stored at −20°C. For immunostaining, slides were rinsed with PBS and blocked for 1 h at room temperature with PBS containing 3% BSA. Subsequently, slides were incubated at 4°C overnight with a primary rabbit anti-mouse active caspase-3 antibody (1:1000) (R&D Systems GmbH, Wiesbaden, Germany) in PBS/3% BSA. After rinsing with PBS, binding sites were detected with a secondary Cy 3-conjugated goat anti-rabbit antibody (1:1000) (Jackson ImmunoResearch Laboratories/Dianova, Hamburg, Germany) for 1 h at room temperature. After prolonged rinsing with PBS, slides were coverslipped using PBS/glycerol, pH 8.6. Negative controls were performed without the primary antibody in presence of the secondary Cy 3-conjugated antibody. To block 80 to 100% of nonspecific binding, slides were incubated in 0.1% bovine serum albumin in PBS for 30 min before staining.

Histology. Zamboni-fixed liver tissue was embedded in paraffin and 8-μm-thick sections were randomly taken throughout the whole organ and stained with H&E using a standard protocol. Sections were screened for necroinflammation as well as for hyperchromatic and condensed hepatocyte nuclei, indicating apoptosis.

Immunocytochemistry and Flow Cytometric Analysis with Primary Hepatocytes. Hepatocytes (106), isolated by the two-step collagenase perfusion method of Seglen (1973) were fixed and permeabilized using CytoFix/CytoPerm Plus (BD Biosciences, Heidelberg, Germany) according to Santoni et al. (2002), rinsed with the washing buffer of the CytoFix/CytoPerm Plus kit, and blocked for 1 h.
at room temperature with washing buffer containing 3% BSA, followed by incubation with the goat anti-NK-1R polyclonal antibody directed against the amino terminus region of human NK-1R (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) at 1:50 dilution. After 1 h at 4°C, cells were washed three times and then labeled with Cy 3-conjugated rabbit anti-goat antibody (1/100 dilution in 3% BSA in washing buffer; Jackson ImmunoResearch/Dianova). Unstained hepatocytes or hepatocytes incubated either with only the primary or the secondary antibody were used as negative controls. After prolonged washing with washing buffer, cells were diluted in 500 μl of 2% paraformaldehyde. Then 100 μl were spun on slides and coverslipped using PBS/glycerol, pH 8.6; 200 μl was added to 200 μl of 0.2 M EDTA and 600 μl of PBS for flow cytometric analysis. To dispose of aggregated hepatocytes, cells were filtered through a 100-μm cell strainer (BD Biosciences Discovery Labware, Bedford, MA), and the suspension was allowed to settle out for 5 min. Then 600 μl of supernatant was used for flow cytometric analysis using a FACScan flow cytometer (BD Biosciences). Data were recorded and analyzed using the BD CellQuest software provided with the flow cytometer, and WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA).

Confocal Laser Scanning Microscopy. Liver cryostat sections or hepatocytes, prepared as described above, were analyzed with a confocal laser scanning microscope (Pascal attached to Axiowert 100M; Carl Zeiss, Oberkochen, Germany). Confocal single optical sections were taken on a 40× oil immersion lens. Images were formatted as TIFF and adjusted for contrast and brightness using Adobe Photoshop 7.0.

Statistical Analysis. The results were analyzed using Student’s t test if two groups were compared or by analysis of variance followed by the Dunnett test if more groups were tested against a control group. If variances were inhomogeneous in the Student’s t test, the results were analyzed using the Welch test. All data in this study are expressed as a mean ± S.E.M. p values less than or equal to 0.05 were considered significant.

Results

Role of Capsaicin-Sensitive Primary Afferent Neurons and SP for Apoptotic Liver Damage. We have described previously that permanent depletion of primary afferent neurons by neonatal capsaicin treatment prevented immune-mediated liver injury in mice by inhibition of expression of proinflammatory cytokines (Tiegs et al., 1999; Bang et al., 2003). This study was intended to prove whether these nerve fibers are also critical for death receptor-mediated apoptosis of parenchymal liver cells in vivo, which is observed in our models of immune-mediated liver damage (Schumann and Tiegs, 1999). For this purpose, we treated 7- to 8-week-old mice, which had received either capsaicin or solvent at 3 days after birth, either with activating anti-CD95 mAb or with recombinant TNFα in combination with GalN. As shown in Fig. 1A, capsaicin-pretreated mice were protected from liver damage as assessed by significantly reduced levels of plasma transaminase activities 8 h after anti-CD95 mAb treatment. Similar results were obtained when we measured active caspase-3, which indicates apoptosis. We have shown previously that the release of transaminases as well as the increase of intracellular caspase-3 activity is close to maximum levels at 8 h after anti-CD95 mAb injection to mice (Sass et al., 2003). The increase of anti-CD95 mAb induced caspase-3 activity, as measured in liver homogenates using a colorimetric assay, was significantly attenuated in capsaicin-pretreated mice (Fig. 1A). Hepatocellular activation of caspase-3 was detectable in liver slices of anti-

CD95 mAb treated mice by immunofluorescence staining using an antibody specific for active caspase-3, which was abolished in mice depleted from primary afferent neurons (Fig. 1B). The reduction of plasma transaminase and
caspase-3 activities in capsaicin pretreated, anti-CD95 mAb treated mice was reversible by intraperitoneal injection of SP immediately after induction of apoptosis (Fig. 1, A and B). Liver histopathology revealed severe apoptotic liver damage upon anti-CD95 mAb application as indicated by numerous hepatocytes with condensed nuclei (Fig. 2B). These changes were drastically reduced by depletion of primary afferents by neonatal capsaicin treatment (Fig. 2C) and reversed by exogenous SP application (Fig. 2D). Capsaicin-induced permanent depletion of primary afferent neurons also significantly reduced apoptotic liver damage induced by TNFα in GalN-sensitized mice (GalN/TNFα: ALT 7544 ± 2121 U/l, AST 9941 ± 2495 U/l; capsaicin pretreatment plus GalN/TNFα: ALT 278 ± 68 U/l*, AST 749 ± 223 U/l*, *p ≤ 0.05 versus GalN/TNFα treatment of solvent-pretreated mice).

**NK-1 Receptor Expression in Mouse Hepatocytes.** The ability of SP to reverse the antia apoptotic effect of C-fiber depletion in the CD95 model suggests that activation of its prime receptor, i.e., NK-1R, might aggravate CD95-induced apoptosis. We have described previously by quantitative reverse transcription-polymerase chain reaction analysis, proof of sequence homology to the NK-1R derived from spinal cord, and immunocytochemistry of tissue slices that the NK-1R is expressed in the mouse liver (Bang et al., 2003). To directly show its expression on murine parenchymal liver cells, we performed NK-1R immunofluorescence staining of freshly isolated primary mouse hepatocytes and detected the receptor either by confocal laser scanning microscopy (Fig. 3A) or by fluorescence-activated cell sorting analysis (Fig. 3B). In addition to our results obtained by immunofluorescence staining of liver slices (Bang et al., 2003), these results indicate that the NK-1R is expressed by mouse hepatocytes.

**Dose-Dependent Protection of NK-1 Receptor Antagonists against Apoptotic Liver Injury Induced by Either Anti-CD95 mAb or GalN/TNFα.** Because SP injection induced caspase-3 activation in the liver and an increase of plasma transaminase activities in anti-CD95 mAb-treated mice that had been depleted from primary sensory neurons, we wondered whether antagonists of the NK-1R would prevent apoptotic liver damage. The specific NK-1R antagonists CP-96,345 and L-733,060, respectively, have previously been shown to prevent immune-mediated liver injury by exerting an anti-inflammatory effect (Bang et al., 2003). Pretreatment of mice with one of both receptor antagonists 30 min before administration of either anti-CD95 mAb or GalN/TNFα, dose dependently reduced plasma transaminase activities and caspase-3 activities in liver homogenates 8 h after the apoptotic challenge (Fig. 4). Similar to the anti-CD95 mAb model, intravenous administration of TNFα to GalN-sensitized mice has been shown to induce high levels of plasma transaminase as well as of intrahepatic caspase-3 activities 8 h after intervention (Sass et al., 2003; Tiegs et al., 1989; Künstle et al., 1999). Both NK-1R antagonists showed a significant protective effect at this time point at doses of 5 to 10 mg/kg (Fig. 4). Protection from both anti-CD95 mAb and GalN/TNFα induced liver damage by both NK-1R antagonists was also proved by histopathology (Fig. 2, E–I). Together, NK-1R antagonists provide protection from apoptotic liver damage that is comparable with their anti-inflammatory potential observed in immune-mediated liver injury (Bang et al., 2003). The protective potential of these substances might explain the cytokine-suppressive and antiapoptotic effect of permanent depletion of primary sensory neurons by capsaicin.

**Discussion**

The present study demonstrates that permanent depletion of primary afferent nerve fibers by neonatal capsaicin treatment as well as antagonists of the NK-1R protected mice from death receptor-mediated apoptotic liver damage. The NK-1R is the high-affinity receptor for SP, i.e., the prime proinflammatory neuropeptide of primary afferent neurons (Harrison and Geppetti, 2001), Both the TNFα/TNFR1 and...

Fig. 2. Histopathological assessment of liver injury in the various models and its protection by neonatal capsaicin or NK-1R antagonist pre-treatment. Livers were excised 8 h after treatment with either anti-CD95 mAb or GalN/TNFα. H&E-stained liver sections from anti-CD95 mAb-treated mice (B) showed severe signs of apoptosis, in particular condensed hepatocyte nuclei (c). Upon GalN/TNFα treatment (G), infiltrates of granulocytes (g) were prominent besides apoptotic hepatocytes showing hyperchromatic (h) and condensed nuclei. In sections from mice pretreated with neonatal capsaicin (C), 5 mg/kg CP 96,345 (E and H), or 5 mg/kg L-733,060 (F and I), these signs of liver injury were minimal and almost comparable with the saline-treated control (A). Exogenous administration of SP dramatically reversed the protection by neonatal capsaicin from anti-CD95 mAb-induced liver injury (D).
the CD95L/CD95 system are activated during an immune response to control infection and to regulate immunity, respectively. In the liver, activation of TNFR1 or CD95 induces apoptosis of hepatocytes and liver damage (Ogasawara et al., 1993; Leist et al., 1996). Both death receptors have been shown to mediate liver injury in our mouse models of immune-mediated hepatitis (Schumann and Tieg, 1999, Seino et al., 1997). We have shown previously that primary afferent neurons as well as the NK-1R are critical for the production of TNFα in the liver (Bang et al., 2003). The NK-1R antagonists CP-96,345 and L-733,060, respectively, significantly reduced intrahepatic expression of TNFα as well as TNFα plasma concentrations induced by administration of LPS to GalN-sensitized mice. Moreover, they also inhibited the production of IFNγ as well as edema formation and granulocyte infiltration in the liver. Hence, our previous study showing an anti-inflammatory effect of NK-1R antagonists in the liver is consistent with other reports showing successful treatment of inflammatory disease in skin, respiratory and gastrointestinal tract (Quartara and Maggi, 1998; Harrison and Gepetti, 2001) with this class of substances.

At present, NK-1R antagonists have been recognized for treatment of human disease because of their potent antidepressant and antiemetic effects (Rupniak and Kramer, 1999). The first high-affinity nonpeptide NK-1 receptor antagonist, CP-96,345, was described by Snider et al. (1991). CP-96,345 was shown to be pharmacologically active in rodents at doses of 5 to 15 mg/kg given either i.p., i.v., or s.c. (Perretti et al., 1993; Veronesi et al., 1995; Costello et al., 1998; Lin et al., 2001). More recently, L-733,060 has been described to inhibit the formalin paw late phase at doses of 0.1 to 10 mg/kg when given intravenously (Rupniak et al., 1996). In our study, both receptor antagonists significantly inhibited caspase-3 activation and liver damage, i.e., release of transaminases, at the same doses range (Fig. 3). Interestingly, in the anti-CD95 mAb as well as in the GalN/TNFα model, L-733,060 inhibited caspase-3 activation and liver damage at lower doses (5 and 10 mg/kg) compared with its cytokine regulatory effect observed in the GalN/LPS model at a dose of 20 mg/kg (Bang et al., 2003). Hence, it seems that NK-1R agonists, e.g., its major ligand SP, have a substantial inhibitory effect on hepatocellular apoptosis in vivo by affecting CD95 and TNFα induced downstream processes such as apoptotic signaling and/or endothelial cell disruption (see below).

Both pro- and antiapoptotic activities have been attributed to SP. In cells originating from the bone marrow, for example, in thymocytes and granulocytes, SP seems to exert antiapoptotic effects, whereas NK-1R antagonists act proapoptotic (Dimri et al., 2000; Bockmann et al., 2001; Santoni et al., 2002). Likewise, SP also promotes proliferation and prevents apoptosis in cancer cells (Heasley, 2001; DeFea et al., 2000; Friess et al., 2003). However, either NK-1R antagonists or disruption of the preprotachykinin A gene, which encodes SP and neurokinin A, have been shown to prevent kainate-induced seizures and neuronal apoptosis associated with caspase-3 activation in vivo (Zachrisson et al., 1998; Liu et al., 1999). In our present study, we obtained indirect evidence for a proapoptotic effect of SP on hepatocellular apoptosis in vivo by showing a protective effect of NK-1R antagonists against CD95- and TNFR1-mediated caspase-3 activation in the liver. Moreover, injection of SP to anti-CD95 mAb treated mice, which have been chemically depleted from their primary afferent nerve fibers, restored hepatocellular activity of caspase-3 (Fig. 1B). This experiment clearly demonstrated that SP was able to potentiate death receptor-induced hepatocellular apoptosis in vivo. It remains to be elucidated, however, whether this effect was direct or mediated by other factors. Although mouse hepatocytes express the NK-1R (Bang et al., 2003; this study), coincubation of primary mouse hepatocytes with SP failed to aggravate anti-CD95 mAb induced apoptosis and cell lysis (data not shown). This might have been due to partial disruption of the receptor by the cell

Fig. 3. Expression of the NK-1R by mouse hepatocytes. Immunofluorescence staining of the expression of the receptor on freshly isolated hepatocytes with a goat anti-human NK-1R polyclonal antibody and a secondary Cy 3-conjugated rabbit anti-goat IgG was detected by confocal laser scanning microscopy (A) or fluorescence-activated cell sorting analysis (B). Unstained hepatocytes, or hepatocytes incubated either with only the primary or the secondary antibody were used as negative controls.
isolation procedure, i.e., by collagenase infusion. However, SP might have also exerted indirect effects toward CD95-induced hepatocellular apoptosis in vivo. For example, SP, by binding to its receptor on endothelial cells, might have increased vascular permeability (Bowden et al., 1994; Harrison and Geppetti, 2001), thereby facilitating the access of anti-CD95 antibodies to liver parenchymal cells. Moreover, administration of activating anti-CD95 mAb to mice results in apoptosis of hepatocytes as well as of hepatic sinusoidal endothelial cells (Janin et al., 2002), thereby inducing severe sinusoidal perfusion failure (Wanner et al., 1999). Hence, the disrupted endothelium might have allowed access of vasoactive substances such as SP to its receptors on vascular smooth muscle cells which results, in case of SP, in an increase of cytosolic Ca^{2+} concentrations in these cells and consequently in vasoconstriction. This leads to hepatic microvascular perfusion failure that might have also contributed to the increase of CD95 induced apoptotic liver damage by SP.

Although TNFα, like agonistic anti-CD95 mAb or soluble CD95 ligand, also induces endothelial cell disruption that precedes tissue damage (Haimovitz-Friedman et al., 1997) and that might have been prevented by C-fiber depletion or NK-1R antagonists, injection of SP failed to reverse the protective effect of C-fiber depletion in the GalN/TNFα model (data not shown). The putative difference between the anti-CD95 and the GalN/TNFα model is that TNFα initially stimulates the production of cytokines at the onset of injury, whereas in the CD95 model, inflammation and cytokine production may occur later as a result from tissue damage. Indeed, histopathological evaluation revealed prominent infiltrates of inflammatory cells besides signs of apoptosis in livers from GalN/TNFα-treated mice, whereas apoptotic changes prevailed over signs of inflammation in anti-CD95 mAb-treated animals. The TNFα-induced inflammatory cascade might activate a different response of primary afferent neurons compared to CD95 activation that cannot be substituted by mere SP injection (e.g., release of neurokinin A, which also binds to NK-1R). Alternatively, SP is not the only neuropeptide activating NK-1 receptors on macrophages such as KC (Marriott and Bost, 2000; Bang et al., 2003), thereby probably aggravating TNFα-induced cytokine synthesis in the liver, which has not yet been excluded to contribute to TNFα-induced hepatocellular apoptosis in vivo. Together, it seems possible that hepatocyte apoptosis and liver damage induced by either agonistic CD95 mAb or TNFα are mediated differently by NK-1R agonists such as SP.

In summary, the present and our previous studies (Tiegs et

![Fig. 4. Protection by the NK-1R antagonists CP-96,345 or L-733,060 against anti-CD95 mAb- or GalN/TNFα-induced liver injury in mice. The NK-1R antagonists were administered i.p. at the doses indicated 30 min before injection of either 120 μg/kg anti-CD95 mAb i.v. or recombinant TNFα (6.5 μg/kg) i.v. in combination with 700 mg/kg GalN i.p. ALT, AST, and activated caspase-3 were detected 8 h after injection with anti-CD95 mAb or GalN/TNFα. Data are expressed as mean values ± S.E.M., n = 4; * p < 0.05 versus saline-treated group (left column).]


