

Neurokinin-1 Receptor Antagonists Protect Mice from CD95- and TNF α -Mediated Apoptotic Liver Damage.

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- c) 26 text pages
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- 247 words in the *Abstract*
- 652 words in the *Introduction*
- 1233 words in the *Discussion*
- d) ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate); CP-96,345, (2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-([2-methoxyphenyl]-methyl)-1-azabicyclo(2.2.2.)-octan-3-amine; DTT, dithiothreitol; GalN, D-galactosamine; L-73,060, (2*S*,3*S*)-3-([3,5-bis(trifluoromethyl)phenyl]methoxy)-2-phenylpiperadine; HEPES, *N*-2-Hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; LPS, lipopolysaccharide; mAb, monoclonal antibody; NK-1R, neurokinin-1 receptor; SP, substance P; TNF α , tumor necrosis factor- α ; TNFR1, TNF receptor 1.
- e) Gastrointestinal, Hepatic, Pulmonary, & Renal

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 pro-inflammatory 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 activating 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 (2*S*,3*S*)-*cis*-2-(diphenylmethyl)-N-([2-methoxyphenyl]-methyl)-1-azabicyclo(2.2.2.)-octan-3-amine (CP-96,345) or (2*S*,3*S*)-3-([3,5-bis(trifluoromethyl)phenyl]methoxy)-2-phenylpiperidine (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 TNF α to D-galactosamine-sensitized mice. In conclusion, SP, probably by binding to its receptor on hepatocytes, might aggravate apoptotic signals in these cells. Since NK-1 receptor antagonists not only suppress the pro-inflammatory 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 pro-inflammatory 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 (CGRP) 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 pro-inflammatory transcription factor NF- κ B (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 (Harrison and Geppetti, 2001; Quartara and Maggi, 1998; Kincy-Cain and Bost, 1996).

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 CGRP immunoreactivity in the portal tract of human (Stoyanova and Gulubova, 1998) and rodent (Markus et al., 1998; Tiegs et al., 1999) livers. Immunoreactive nerve fibers were capsaicin-sensitive, i.e. they were completely absent in adult mice that have been treated with capsaicin three days after birth in order 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 non-sensitized 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-1R antagonists prevented inflammatory liver disease by inhibiting NF- κ B activation, by suppressing the synthesis of pro-inflammatory and concomitantly increasing the production of hepatoprotective cytokines (Bang et al., 2003). The tumor necrosis factor α (TNF α)/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 (reviewed in Schümann and Tiegs, 1999; Seino et al., 1997). To shortcut the system, i.e. to directly activate the death receptors TNFR1 or CD95 *in vivo*, administration of recombinant TNF α to GalN-sensitized mice or of activating anti-CD95 monoclonal antibody (mAb) to non-sensitized mice can be used to induce hepatocyte apoptosis, secondary necrosis and liver damage (reviewed in Schümann and Tiegs, 1999; Ogasawara et al., 1993).

Since 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 H et al., 1999), we wondered whether capsaicin-sensitive primary afferent neurons, their transmitter peptide SP as well as the NK-1R are directly involved in apoptotic liver damage induced by death receptor activation *in vivo*. Because the NK-1R 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.

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 NIH, as well as to the legal requirements in Germany. They were maintained under controlled conditions (22°C, 55% humidity, and 12-hour day/night rhythm) and fed a standard laboratory chow.

Dosages and application routes. Capsaicin pretreatment: Newborn BALB/c mice were injected with 100mg/kg capsaicin subcutaneously (s.c.) in a volume of 40 μ L on the third 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 pre-treatment 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 5 wipes in 30 s. Most capsaicin-pretreated animals did not respond at all.

All other reagents were injected in a total volume of 250 μ L per 25 g mouse. Activating anti-mouse CD95 mAb (Jo2; BD/PharMingen/Transduction Laboratories; Heidelberg; Germany) was administered intravenously in pyrogen-free saline at a dose of 120 μ g/kg. SP (Tocris/Biotrend Chemikalien GmbH, Köln, Germany, 5mg/kg, dissolved in pyrogen-free saline) was injected intraperitoneally immediately after Jo2 application. Recombinant murine TNF α 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 (Roth GmbH, Karlsruhe, Germany) in pyrogen-free saline.

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

Analysis of liver enzymes. Hepatocyte damage was assessed 8 hours 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 (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4, 1 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) and 1 mM DTT (dithiothreitol) and analyzed using the colorimetric caspase-3 Assay Kit (Sigma Chemical Co., 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 GSV 1 tissue-embedding medium (Slee Technik GmbH, Mainz, Germany), frozen in 2-methyl-butane (Carl Roth GmbH, Karlsruhe, Germany), and stored at -20°C until use. Cryostat sections of 10 µm were thawed on glass slides, air-dried, fixed for 10 minutes 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 hour 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 Immuno Research Laboratories, INC./Dianova, Hamburg, Germany) for 1 hour 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.

Histology. Zamboni-fixed liver tissue was embedded in paraffin and 8 μm thick sections were randomly taken throughout the whole organ and stained with hematoxylin and eosin (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. 10^6 hepatocytes, 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 hour 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 hour at 4°C, cells were washed three times and then labeled with Cy 3-conjugated rabbit anti-goat antibody (Jackson/Dianova, Hamburg, Germany, 1/100 dilution in 3% BSA in washing buffer). Unstained hepatocytes, or hepatocytes incubated either with only the primary or the secondary antibody were used as negative controls. After prolonged rinsing with washing buffer, cells were diluted in 500 μL 2% paraformaldehyde. 100 μL were spun on slides and coverslipped using PBS/glycerol, pH 8.6; 200 μL were added to 200 μL 0,2M EDTA and 600 μL PBS for flow cytometric analysis. To dispose of aggregated hepatocytes, cells were filtered through a 100 μm cell strainer

(FALCON/Becton Dickinson Labware) and the suspension was allowed to settle out for 5 minutes. 600 μ L of supernatant were used for flow cytometric analysis using a Becton-Dickinson FACScanTM Flow Cytometer (BD Biosciences, Heidelberg, Germany). Data were recorded and analyzed using the BD CellQuestTM software provided with the flow cytometer, and WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA).

Confocal laser scanning microscopy. Liver cryostate sections or hepatocytes, prepared as described above, were analyzed with a confocal laser scanning microscope (Zeiss Pascal attached to Axiovert 100M, Carl Zeiss, Oberkochen, Germany). Confocal single optical sections were taken on a 40 x 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 ANOVA 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 Welsh test. All data in this study are expressed as a mean \pm SEM. 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 pro-inflammatory 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 (Schümann and Tiegs, 1999). For this purpose we treated 7 to 8 weeks old mice, which had received either capsaicin or solvent at 3 days after birth, either with activating anti-CD95 mAb or with recombinant TNF α in combination with GalN. As shown in Figure 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 intrahepatic caspase-3 activity are close to maximum levels at 8 h following 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. 1A 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 TNF α in GalN-sensitized mice (GalN/TNF α : ALT 7544 \pm 2121 U/L, AST 9941 \pm 2495 U/L; capsaicin pretreatment *plus* GalN/TNF α : ALT 278 \pm 68* U/L, AST 749 \pm 223* U/L, *p \leq 0.05 vs GalN/TNF α treatment of solvent pretreated mice).

NK-1 receptor expression in mouse hepatocytes. The ability of SP to reverse the anti-apoptotic effect of C-fiber depletion in the CD95 model suggests that activation of its prime receptor, i.e. the NK-1 receptor (NK-1R), might aggravate CD95-induced apoptosis. We have described previously by quantitative RT-PCR 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). In order 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 FACS 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/TNF α . Since SP injection induced caspase-3 activation in the liver and an increase of plasma transaminase activities in anti-CD95 mAb-treated mice which 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/TNF α , 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 TNF α to GalN-sensitized mice has been shown to induce high levels of plasma transaminase as well as of intrahepatic caspase-3 activities 8h 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/TNF α induced liver damage by both NK-1R antagonists was also proved by histopathology (Fig. 2E-I). Taken together, NK-1R antagonists provide protection from apoptotic liver damage which is comparable to 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 anti-apoptotic 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 pro-inflammatory neuropeptide of primary afferent neurons (Harrison and Geppetti, 2001). Both, the TNF α /TNFR1 and the CD95L/CD95 system are activated during an immune response in order 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 (Schümann and Tiegs, 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 TNF α in the liver (Bang et al., 2003). The NK-1R antagonists CP-96,345 and L-733,060, respectively, significantly reduced intrahepatic expression of TNF α as well as TNF α plasma concentrations induced by administration of LPS to GalN-sensitized mice. Moreover, they also inhibited the production of IFN γ 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 Geppetti, 2001) with this class of substances.

At present, NK-1R antagonists have been recognized for treatment of human disease because of their potent anti-depressant and anti-emetic effects (Rupiniak and Kramer, 1999). The first high-affinity nonpeptide NK-1 receptor antagonist, CP-96,345, was described by Snider et al. in 1991. CP-96,345 was shown to be pharmacologically active in rodents at doses of 5-15 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/TNF α model, L-733,060 inhibited caspase-3 activation and liver damage at lower doses (5 and 10 mg/kg) compared to its cytokine regulatory effect observed in the GalN/LPS model at a dose of 20mg/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 TNF α induced downstream processes such as apoptotic signaling and/or endothelial cell disruption (see below).

Both, pro- and anti-apoptotic activities have been attributed to SP. In cells originating from the bone marrow, for example in thymocytes and granulocytes, SP seems to exert anti-apoptotic effects whereas NK-1R antagonists act pro-apoptotic (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 H, 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 pro-apoptotic 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, and this study), co-incubation 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 isolation procedure, i.e. by collagenase infusion. However, SP might have also exerted indirect effects towards 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 which might have also contributed to the increase of CD95 induced apoptotic liver damage by SP.

Although $TNF\alpha$, like agonistic anti-CD95 mAb or soluble CD95 ligand, also induces endothelial cell disruption which precedes tissue damage (Haimovitz-Friedman et al., 1997) and which 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/ $TNF\alpha$ model (data not shown). The putative difference between the anti-CD95 and the GalN/ $TNF\alpha$ model is that $TNF\alpha$ 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/ $TNF\alpha$ -treated mice whereas apoptotic

changes prevailed over signs of inflammation in anti-CD95 mAb treated animals. The TNF α induced inflammatory cascade might activate a different response of primary afferent neurons compared to CD95 activation which 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 NK1 receptors on macrophages such as KC (Marriott I and Bost KL, 2000; Bang et al., 2003) thereby probably aggravating TNF α -induced cytokine synthesis in the liver, which has not yet been excluded to contribute to TNF α induced hepatocellular apoptosis *in vivo*. Taken together, it seems possible that hepatocyte apoptosis and liver damage induced by either agonistic CD95 mAb or TNF α are mediated differently by NK-1R agonists such as SP.

In summary, the present and our previous studies (Tiegs et al., 1999; Bang et al., 2003) indicate that inflammatory cytokine-mediated apoptotic liver injury is affected by neuropeptides released from peripheral endings of capsaicin-sensitive primary afferents. NK-1R agonists such as SP seem to be major players in this scenario by up-regulating the pro-inflammatory cytokine response, by sensitizing hepatocytes towards death receptor-mediated apoptosis either directly or by more indirect modes of action such as alterations of hepatic blood flow. Since NK-1R antagonists 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 suitable for treatment of cytokine-mediated liver disease such as autoimmune hepatitis, ischemia/reperfusion injury during liver transplantation (Jaeschke H, 2003) or toxic hepatitis (Schümann and Tiegs, 1999).

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References

- Bang R, Sass G, Kiemer AK, Vollmar AM, Neuhuber WL, and Tiegs G (2003) Neurokinin-1 receptor antagonists CP-96,345 and L-733,060 protect mice from cytokine-mediated liver injury. *J Pharmacol Exp Ther* 305: 31-39.
- Bergmeyer HU (1984) Methods of enzymatic analysis. 3rd edition. Volume 82. Verlag Chemie. Weinheim, Germany. 416–456.
- Bockmann S, Seep J, and Jonas L (2001) Delay of neutrophil apoptosis by the neuropeptide substance P: involvement of caspase cascade. *Peptides* 22:661-670.
- Bowden JJ, Garland AM, Baluk P, Lefevre P, Grady EF, Vigna SR, Bunnett NW, and McDonald DM (1994) Direct observation of substance P-induced internalization of neurokinin 1 (NK1) receptors at sites of inflammation. *Proc Natl Acad Sci U S A* 91:8964-8968.
- Bracci-Laudiero L, Aloe L, Stenfors C, Theodorsson E, and Lundeberg T (1998) Development of systemic lupus erythematosus in mice is associated with alterations of neuropeptide concentrations in inflamed kidneys and immunoregulatory organs. *Neurosci Lett* 248:97-100.
- Casini A, Lippe IT, Evangelista S, Geppetti P, Santicoli P, Urso C, Paglierani M, Maggi CA, and Surrenti C (1990) Effect of sensory denervation with capsaicin on liver fibrosis induced by common bile duct ligation in rat. *J Hepatol* 11:302-312.
- Costello RW, Fryer AD, Belmonte KE, and Jacoby DB (1998) Effects of tachykinin NK1 receptor antagonists on vagal hyperreactivity and neuronal M2 muscarinic receptor function in antigen challenged guinea-pigs. *Br J Pharmacol* 124:267-276.
- DeFea KA, Vaughn ZD, O'Bryan EM, Nishijima D, Dery O, and Bunnett NW (2000) The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta-arrestin-dependent scaffolding complex. *Proc Natl Acad Sci U.S.A.* 97:11086-11091.

- del Rio G, Castro-Obregon S, Rao R, Ellerby HM, and Bredesen DE (2001) APAP, a sequence-pattern recognition approach identifies substance P as a potential apoptotic peptide. *FEBS Lett* 494:213-219.
- Dimri R, Sharabi Y, and Shoham J (2000) Specific inhibition of glucocorticoid-induced thymocyte apoptosis by substance P. *J Immunol* 164:2479-2486.
- Friess H, Zhu Z, Liard V, Shi X, Shrikhande SV, Wang L, Lieb K, Korc M, Palma C, Zimmermann A, Reubi JC, and Buchler MW (2003) Neurokinin-1 receptor expression and its potential effects on tumor growth in human pancreatic cancer. *Lab Invest* 83:731-742.
- Haimovitz-Friedman A, Cordon-Cardo C, Bayoumy S, Garzotto M, McLoughlin M, Gallily R, Edwards CK 3rd, Schuchman EH, Fuks Z, and Kolesnick R (1997) Lipopolysaccharide induces disseminated endothelial apoptosis requiring ceramide generation. *J Exp Med* 186:1831-1841.
- Harrison S and Geppetti P (2001) Substance P. *Int J Biochem Cell Biol* 33:555-576.
- Heasley LE (2001) Autocrine and paracrine signaling through neuropeptide receptors in human cancer. *Oncogene* 20:1563-1569.
- Holzer P (1988) Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neurosci* 24:739-768.
- Jaeschke H (2003) Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. *Am J Physiol Gastrointest Liver Physiol* 284:G15-G26.
- Janin A, Deschaumes C, Daneshpouy M, Estaquier J, Micic-Polianski J, Rajagopalan-Levasseur P, Akarid K, Mounier N, Gluckman E, Socie G, and Ameisen JC (2002) CD95 engagement induces disseminated endothelial cell apoptosis in vivo: immunopathologic implications. *Blood* 99:2940-2947.

- Kincy-Cain T and Bost KL (1996) Increased susceptibility of mice to Salmonella infection following *in vivo* treatment with the substance P antagonist, spantide II. *J Immunol* 157:255-264.
- Künstle G, Hentze H, Germann P-G, Tiegs G, Meergans T, and Wendel A (1999) Concanavalin A hepatotoxicity in mice: tumor necrosis factor-mediated organ failure independent of caspase-3-like protease activation. *Hepatology* 30:1241-1251.
- Leist M, Gantner F, Kunstle G, Bohlinger I, Tiegs G, Bluethmann H and Wendel A (1996) The 55-kD tumor necrosis factor receptor and CD95 independently signal murine hepatocyte apoptosis and subsequent liver failure. *Mol Med* 2:109-124.
- Lin YS, Ho CY, Tang GJ, and Kou YR (2001) Alleviation of wood smoke-induced lung injury by tachykinin receptor antagonist and hydroxyl radical scavenger in guinea pigs. *Eur J Pharmacol* 425:141-148.
- Liu H, Cao Y, Basbaum AI, Mazarati AM, Sankar R, and Wasterlain CG (1999) Resistance to excitotoxin-induced seizures and neuronal death in mice lacking the preprotachykinin A gene. *Proc Natl Acad Sci U S A* 96:12096-12101.
- Lotz M, Vaughan JH, and Carson DA (1988) Effect of neuropeptides on production of inflammatory cytokines by human monocytes. *Science* 241:1218-1221.
- Markus AM, Kockerling F and Neuhuber WL (1998) Close anatomical relationship between nerve fibers and MHC class II-expressing dendritic cells in the rat liver and extrahepatic bile duct. *Histochem Cell Biol* 109:409-415.
- Marriott I, Bost KL (2000) IL-4 and IFN- γ up-regulate substance P receptor expression in murine peritoneal macrophages. *J Immunol* 165:182-91.
- Marriott I, Mason MJ, Elhofy A, and Bost KL (2000) Substance P activates NF- κ B independent of elevations in intracellular calcium in murine macrophages and dendritic cells. *J Neuroimmunol* 102:163-171.

- Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, and Nagata S (1993) Lethal effect of the anti-Fas antibody in mice. *Nature* 364:806-809.
- Perretti M, Ahluwalia A, Flower RJ, and Manzini S (1993) Endogenous tachykinins play a role in IL-1-induced neutrophil accumulation: involvement of NK-1 receptors. *Immunology* 80:73-77.
- Quartara L and Maggi CA (1998) The tachykinin NK1 receptor. Part II: distribution and pathophysiological roles. *Neuropeptides* 32:1-49.
- Rameshwar P, Ganea D, and Gascon P (1994) Induction of IL-3 and granulocyte-macrophage colony-stimulating factor by substance P in bone marrow cells is partially mediated through the release of IL-1 and IL-6. *J Immunol* 152:4044-4054.
- Rupniak NM, Carlson E, Boyce S, Webb JK, and Hill RG (1996) Enantioselective inhibition of the formalin paw late phase by the NK1 receptor antagonist L-733,060 in gerbils. *Pain* 67:189-195.
- Rupniak NM and Kramer MS (1999) Discovery of the antidepressant and anti-emetic efficacy of substance P receptor (NK1) antagonists. *Trends Pharmacol Sci* 20:485-490.
- Santoni G, Amantini C, Lucciarini R, Pompei P, Perfumi M, Nabissi M, Morrone S, and Piccoli M (2002) Expression of substance P and its neurokinin-1 receptor on thymocytes: functional relevance in the regulation of thymocyte apoptosis and proliferation. *Neuroimmunomodulation* 10:232-246.
- Sass G, Soares MCP, Yamashita K, Seyfried S, Zimmermann WH, Eschenhagen T, Kaczmarek E, Ritter T, Volk HD, and Tiegs G (2003) Heme oxygenase-1 and its reaction product carbon monoxide prevent inflammation related apoptotic liver damage in mice. *Hepatology* in press.

- Schümann J and Tiegs G (1999) Pathophysiological mechanisms of TNF during intoxication with natural or man-made toxins. *Toxicology* 138:103-126.
- Seglen PO (1973) Preparation of rat liver cells. 3. Enzymatic requirements for tissue dispersion. *Exp Cell Res* 82:391-398.
- Seino K, Kayagaki N, Takeda K, Fukao K, Okumura K, and Yagita H (1997) Contribution of Fas ligand to T cell-mediated hepatic injury in mice. *Gastroenterology* 113: 1315-1322.
- Snider RM, Constantine JW, Lowe III JA, Longo KP, Lebel WS, Woody HA, Drozda SE, Desai MC, Vinick FJ, Spencer RW, and Hess HJ (1991) A potent nonpeptide antagonist of the substance P (NK₁) receptor. *Science* 251:435-437.
- Stoyanova II and Gulubova MV (1998) Peptidergic nerve fibres in the human liver. *Acta Histochem* 100:245-256.
- Tiegs G, Wolter M, and Wendel A (1989) Tumor necrosis factor is a terminal mediator in galactosamine/endotoxin-induced hepatitis in mice. *Biochem Pharmacol* 38:627-631.
- Tiegs G, Bang R and Neuhuber WL (1999) Requirement of peptidergic sensory innervation for disease activity in murine models of immune hepatitis and protection by β -adrenergic stimulation. *J Neuroimmunol* 96:131-143.
- Veronesi B, Sailstad DM, Doerfler DL, and Selgrade M (1995) Neuropeptide modulation of chemically induced skin irritation. *Toxicol Appl Pharmacol* 135:258-267.
- Wanner GA, Mica L, Wanner-Schmid E, Kolb SA, Hentze H, Trentz O, and Ertel W (1999) Inhibition of caspase activity prevents CD95-mediated hepatic microvascular perfusion failure and restores Kupffer cell clearance capacity. *FASEB J* 13:1239-48
- Zachrisson O, Lindefors N, and Brene S (1998) A tachykinin NK1 receptor antagonist, CP-122,721-1, attenuates kainic acid-induced seizure activity. *Brain Res Mol Brain Res* 60:291-295.

Footnotes

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Figure legends

Fig. 1: SP aggravates apoptotic liver damage which depends on the presence of capsaicin-sensitive primary afferent neurons. Newborn BALB/c mice were pretreated with 100 mg/kg capsaicin or solvent s.c. at the third day after birth. Seven weeks later they were treated with activating anti-CD95 mAb (120 μ g/kg Jo2) i.v. alone or together with 5 mg/kg SP i.p.

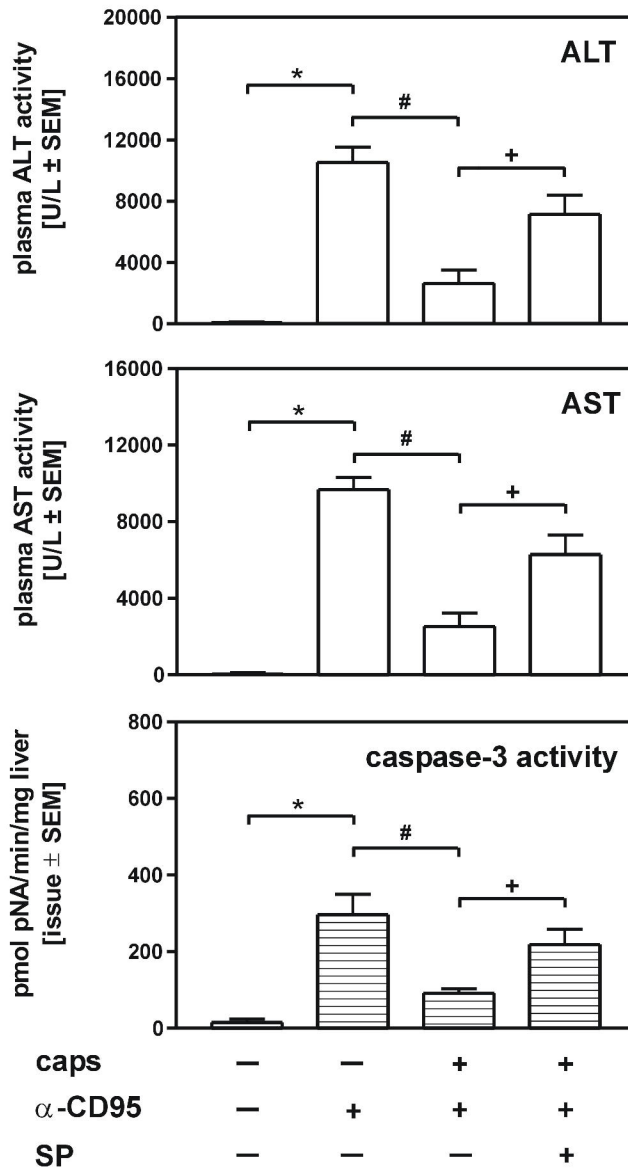
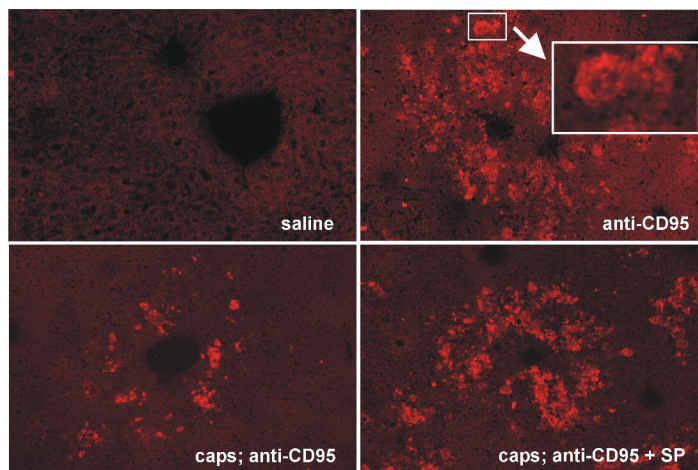
A: ALT, AST and caspase-3 activity were detected 8 h after anti-CD95 mAb injection. Data are expressed as mean values \pm SEM; n = 5; *p \leq 0.05 of the anti-CD95 mAb treated group vs. saline control; n = 5; #p \leq 0.05 of anti-CD95 treated groups: capsaicin non-pretreated control vs. capsaicin pretreated group; +p \leq 0.05 of both capsaicin pretreated groups: anti-CD95 treated group vs. anti-CD95 treated group with additional application of SP.

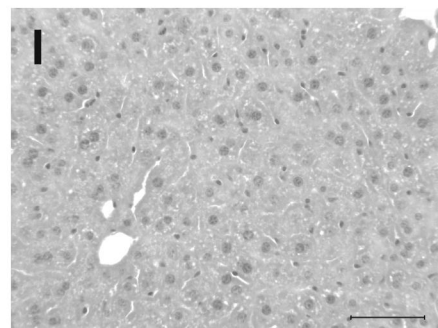
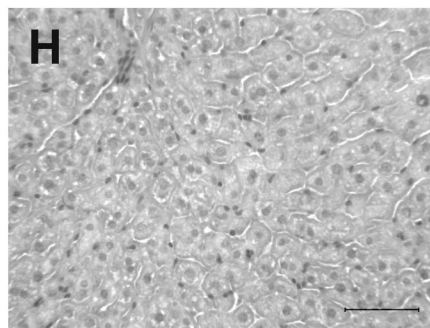
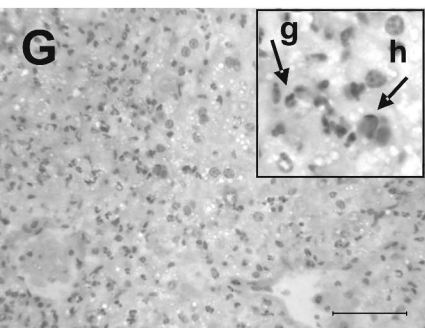
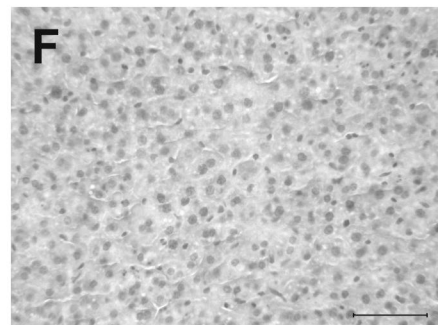
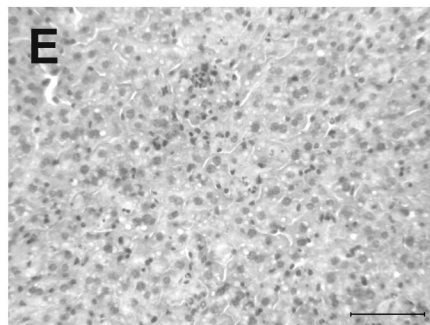
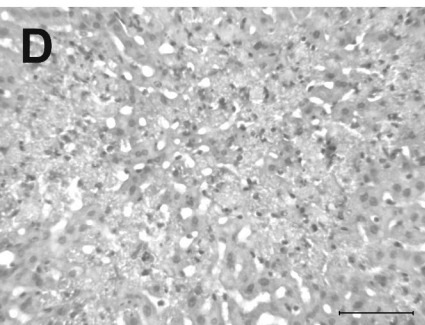
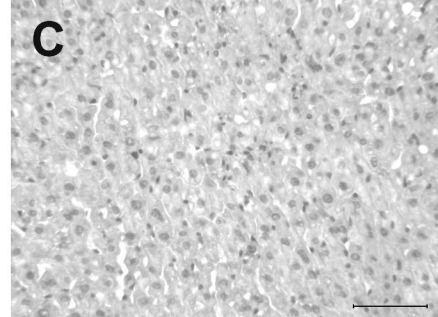
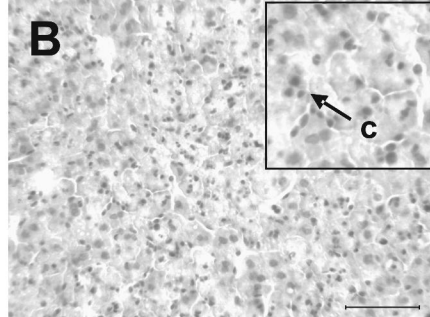
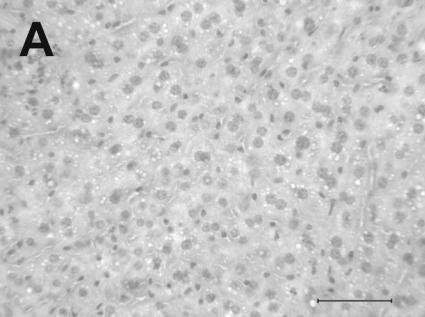
B: Representative liver sections of mice were subjected to immunofluorescence staining of activated caspase-3 and confocal laser scanning microscopy 8 h after anti-CD95 mAb treatment with or without additional SP injection.

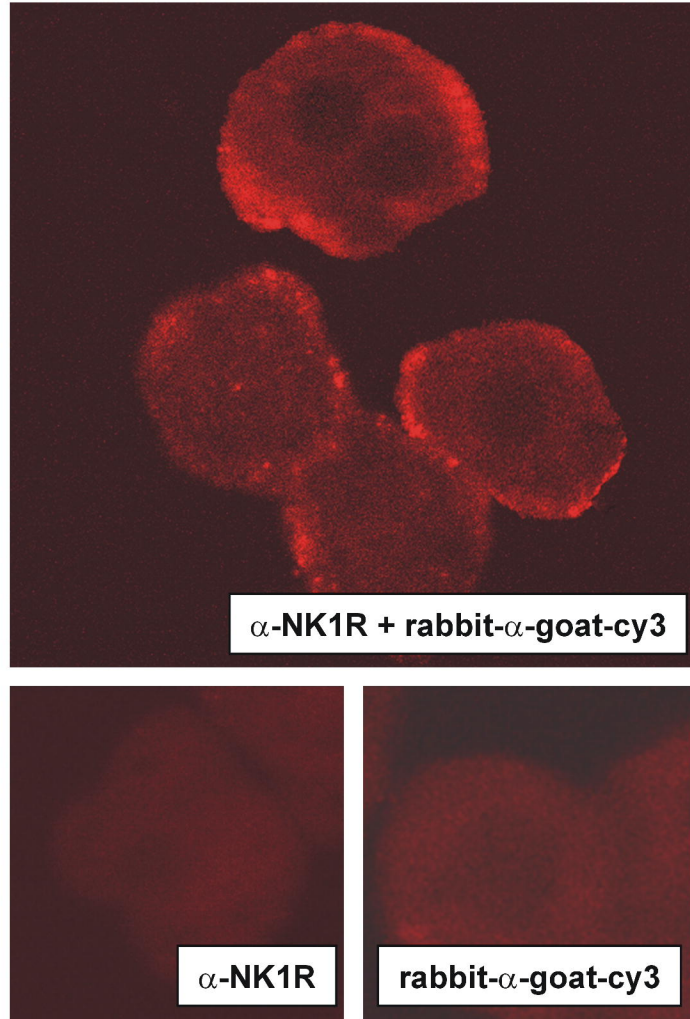
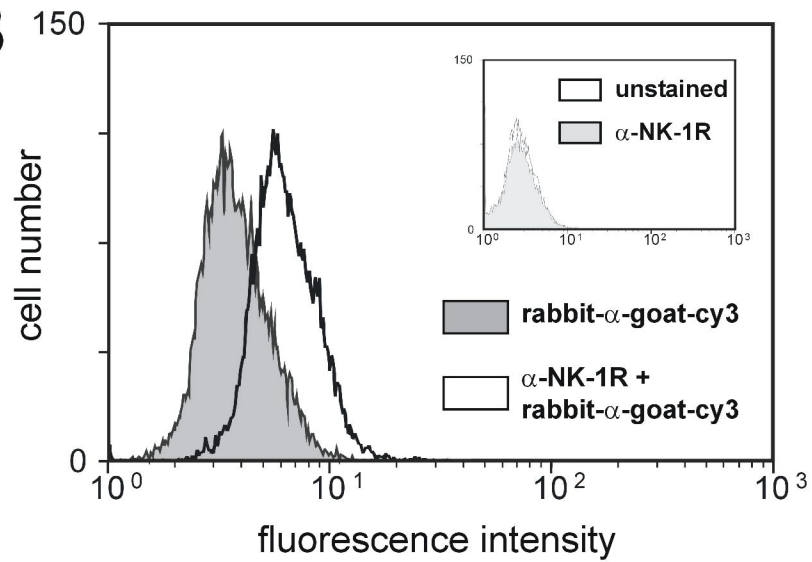
Fig. 2: Histopathological assessment of liver injury in the various models and its protection by neonatal capsaicin or NK-1R antagonist pretreatment. Livers were excised 8 h after treatment with either anti-CD95 mAb or GalN/TNF α . H&E-stained liver sections from anti-CD95 mAb treated mice (B) showed severe signs of apoptosis, in particular condensed hepatocyte nuclei (c). Upon GalN/TNF α 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,H), or 5 mg/kg L-733,060 (F,I) these signs of liver injury were minimal and almost comparable to the saline-treated control (A). Exogenous administration of SP dramatically reversed the protection by neonatal capsaicin from anti-CD95 mAb induced liver injury (D).

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 Ab and a secondary Cy 3 conjugated rabbit anti-goat IgG was detected by confocal laser scanning microscopy (A) or FACS analysis (B). Unstained hepatocytes, or hepatocytes incubated either with only the primary or the secondary antibody were used as negative controls.

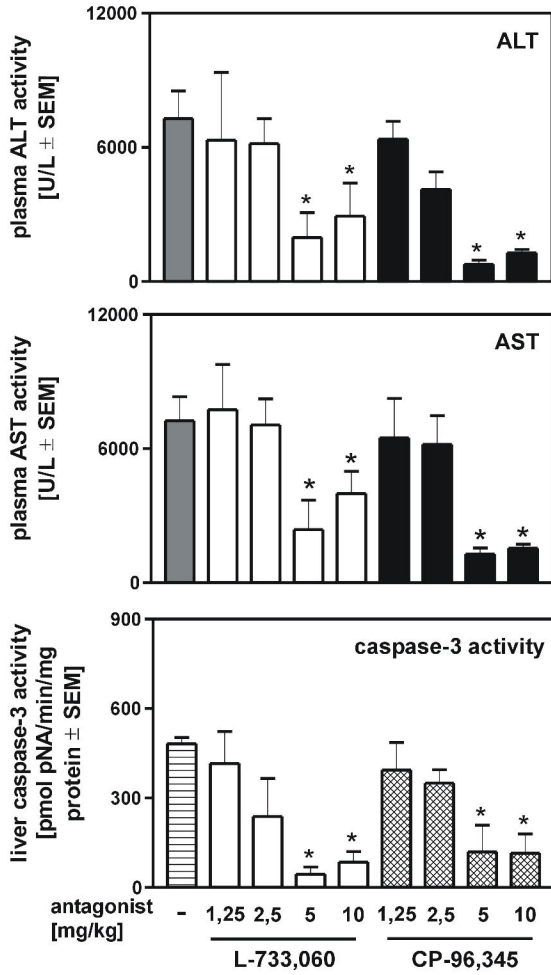
Fig. 4: Protection by the NK-1R antagonists CP-96,345 or L-733,060 against anti-CD95 mAb- or GalN/TNF α -induced liver injury in mice. The NK-1R antagonists were administered i.p. at the doses indicated 30 min prior to injection of either 120 μ g/kg anti-CD95 mAb i.v. or recombinant TNF α (6.5 μ g/kg) 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/TNF α . Data are expressed as mean values \pm SEM; n = 4; *p \leq 0.05 vs. saline treated group (left bar).

A**B**



A**B**

α -CD95



GaIN/TNF α

