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Neuropeptide PACAP in Mouse Liver Ischemia and Reperfusion Injury: Immunomodulation via cAMP-PKA Pathway

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Abstract

Hepatic ischemia and reperfusion injury (IRI), an exogenous antigen-independent local inflammation response, occurs in multiple clinical settings including liver transplantation, hepatic resection, trauma, and shock. The immune system and the nervous system maintain extensive communication, and mount a variety of integrated responses to danger signals through intricate chemical messengers. This study examined the function and potential therapeutic potential of neuropeptide PACAP (pituitary adenylate cyclase-activating polypeptide) in a murine model of partial liver “warm” ischemia (90min) followed by reperfusion. Liver IR readily triggered the expression of intrinsic PACAP and its receptors, whereas the hepatocellular damage was exacerbated in PACAP-deficient mice. Conversely, PACAP27, or PACAP38 peptide monotherapy, which elevates intracellular cyclic adenosine monophosphate - protein kinase A (cAMP-PKA) signaling, protected livers from IRI, as evidenced by diminished serum alanine aminotransferase (ALT) levels and well-preserved tissue architecture. The liver protection rendered by PACAP peptides was accompanied by diminished neutrophil/macrophage infiltration and activation, reduced hepatocyte necrosis/apoptosis, and selectively augmented hepatic IL-10 expression. Strikingly, PKA inhibition readily restored liver damage in otherwise IR-resistant PACAP-conditioned mice. *In vitro*, PACAP treatment not only diminished macrophage TNF- α /IL-6/IL-12 levels in an PKA-dependent manner, but also prevented necrosis and apoptosis in primary mouse hepatocyte cultures.

Conclusion—Our novel findings document the importance of PACAP mediated cAMP-PKA signaling in hepatic homeostasis and cytoprotection *in vivo*. As the enhancement of neural modulation differentially regulates local inflammation and prevents hepatocyte death, these results provide the rationale for novel approaches to manage liver inflammation and IRI in transplant patients.

Keywords

Cyclic Adenosine Monophosphate; Ischemia/Reperfusion Injury; Pituitary Adenylate Cyclase-Activating Polypeptides; Protein kinase A; Toll-like Receptor 4

Hepatic ischemia-reperfusion injury (IRI), an exogenous antigen-independent inflammation response, occurs in multiple clinical settings including liver transplantation, hepatic resection, trauma, and shock (1). Liver IR-mediated local tissue damage combines two phases of ischemia-triggered hypoxic cellular stress, and inflammation-mediated reperfusion injury. Endogenous reactive oxygen species (ROS)-inflicted tissue damage initiates circulatory disturbances and cascade of inflammation responses, leading to the ultimate hepatocyte death. Our group was among the first to document that activation of the sentinel TLR4 signaling is required in the mechanism of liver IRI (2). We then provided evidence that IR-triggered TLR4, primarily on Kupffer cells/macrophages, activates downstream “signature” pro-inflammatory programs, i.e., TNF- α , IFN- β , and CXCL-10 (3,4).

The immune system and the nervous system maintain extensive communication, and mount a variety of integrated responses to danger signals through intricate chemical messengers. The innate immune system provides the first defense line against invading pathogens, through recognition of pathogen-associated molecular patterns (PAMPs), and releasing pro-inflammatory mediators (5). These immune components convey the peripheral message to the brainstem and preoptic area of the anterior hypothalamus, the activate systemic neuroendocrine hypothalamus and regional neural-hormonal–stress response, which amplify local inflammation to eliminate pathogens (6–9). This interplay constitutes an important feedback loop that optimizes, monitors and adjusts innate inflammation by stimulation of efferent vagus nerve activity (6,7). The neural modulation of local inflammation eventually restores the host homeostasis, and return to a resting status (10).

The mammalian nervous system, equipped with neuropeptides and peptide hormones with pro- and anti-inflammatory properties, may directly defend the host from microbial assault (9). Pituitary adenylate cyclase-activating polypeptides (PACAP), a 38-amino-acid neuropeptide (PACAP38) and a C-terminally truncated 27-amino-acid form (PACAP27) originally isolated from ovine hypothalamus (11), belong to the secretin/glucagon/vasoactive intestinal peptide (VIP) family. The PACAP sequence shows a 68% homology with VIP, and was identified as a hypothalamic hormone that stimulates adenylate cyclase in pituitary cells (12). PACAP is expressed throughout the nervous system, adrenal gland, gastrointestinal tract, pancreas and liver (12). Interestingly, PACAP storage/gene expression is found in central (thymus) and peripheral (spleen, lymph nodes) lymphoid organs and some lymphoid cells (13). PACAP exerts its function through three G protein-coupled receptors (GPCRs) (12). These include vasoactive receptors with high affinity for VIP and PACAP, i.e., VPAC1, constitutively expressed in lymphocytes/macrophages, and VPAC2, expressed selectively in stimulated lymphocytes/macrophages (12). The third receptor, PAC1 (pituitary adenylate cyclase-activating polypeptide 1 receptor) favors PACAP (300–1000 fold higher binding affinity than VIP) and macrophages (12). Hepatocytes express all three PACAP receptors (14).

The highly conserved sequence, with wide expression and storage locations, suggest PACAP may affect different physiological functions (15). Indeed, PACAP peptides inhibit LPS-induced TNF- α by increasing activity of cAMP-PKA axis and cAMP response element-binding protein (CREB) (16), and modulating NF- κ B activity (17). We have shown that by differentially regulating local inflammation, activation of cAMP-PKA prevented hepatocyte death (18), whereas others reported that PACAP deficiency resulted in higher susceptibility to retinal ischemic injury (19). These data suggesting therapeutic potential of PACAP neuropeptide warrant confirmation in animal inflammatory disease models.

This study was designed to examine putative therapeutic effects and mechanisms by which PACAP may contribute to liver homeostasis in IR-mediated hepatocellular insult. As stress triggers pro- and anti-inflammatory response by neuropeptides/peptide hormones, we first

determined the function of endogenous PACAP in pathophysiology of liver IRI. The question then arose whether exogenous PACAP can diminish pro-inflammatory response and promote hepatocyte survival. Finally, a key issue as to whether PACAP-induced cAMP-PKA activation is essential for liver homeostasis warrants critical evaluation while considering neural immunomodulation as a novel therapeutic concept in the management of liver inflammation.

Experimental Procedures

Animals

Male 8–12 weeks old wild-type (WT) (Jackson Laboratory, Bar Harbor, ME) and PACAP-deficient mice (20) on a C57BL/6 background (backcrossed for at least twelve generations) were used. Animals were housed in the UCLA animal facility under specific pathogen-free conditions and received humane care according to the criteria outlined in *Guide for the Care and Use of Laboratory Animals* (prepared by the National Academy of Sciences; NIH publication 86–23, revised 1985).

Mouse warm liver IRI model

We have used a mouse model of partial “warm” hepatic IRI (2). In brief, animals were anesthetized, injected with heparin (100U/kg i.p.), and the arterial/portal venous blood supply to the cephalad lobes was interrupted by an atraumatic clip for 90min. Sham-operated mice underwent the same procedure, but without vascular occlusion. In the treatment groups, animals were infused 1h prior to the onset of liver ischemia with a single dose of PACAP27 or PACAP38 neuropeptide (50nmol/mouse i.v., Phoenix Pharmaceuticals, Burlingame, CA) dissolved in PBS. Some recipients were given H-89 (cAMP-PKA inhibitor; 20nmol/mouse i.v., Sigma-Aldrich, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO). Mice were sacrificed at various time-points of reperfusion; liver and serum samples were collected for analysis.

The hepatocellular damage

Serum alanine aminotransferase (sALT) levels were measured by IDEXX Laboratory (Westbrook, ME). Culture medium ALT levels were measured by ALT kit (Stanbio, Boerne, TX). Untreated hepatocyte lysates were used to determine total ALT level. Cell death was expressed as ALT released from treated cells (percentage of the total ALT).

Histopathology

Liver specimens (4µm), stained with hematoxylin and eosin (H&E), were analyzed blindly by modified Suzuki’s criteria (21). Primary mAb against mouse neutrophils Ly-6G (1A8; BD Biosciences, San Jose, CA) and macrophages CD68 (FA-11; AbD Serotec, Raleigh, NC) were used (21). Liver sections were evaluated blindly by counting labeled cells in 10 high-power fields (HPF).

Myeloperoxidase activity assay

The presence of myeloperoxidase (MPO) was used as an index of neutrophil accumulation in the liver (21). One absorbance unit (U) of MPO activity was defined as the quantity of enzyme degrading 1mol peroxide/min at 25°C/gram of tissue.

Quantitative RT-PCR

Quantitative PCR was performed with platinum SYBR green quantitative PCR kit (Invitrogen, Carlsbad, CA) by the Chromo 4 detector (MJ Research, Waltham, MA). Primers to amplify specific gene fragments were published (21). The sequence of PACAP

and PACAP receptor primers is shown (Supplementary Table 1). Target gene expressions were calculated by their ratios to the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT).

Western blots

Western blots were performed with liver proteins (30µg/sample) and rabbit anti-mouse Bcl-2, Bcl-xl, p-IκBα, p-NF-κB p65, and β-actin mAbs (Cell Signaling Technology, Danvers, MA) (21). Relative quantities of protein were determined by densitometer and expressed in absorbance units (AU).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

DNA fragments in liver sections, resulting from oncotic necrosis and apoptosis were detected by TUNEL method (Klenow-FragEL DNA Fragmentation Detection Kit, Calbiochem, La Jolla, CA) (21). TUNEL positive cells were counted in 10 HPF/section under light microscopy (×400).

Caspase-3 activity assay

Caspase-3 activity was performed using Caspase-3 Cellular Activity Assay Kit (Calbiochem). Liver tissue sample and cell lysis were used according to the manufacturer's instruction.

The cAMP/PKA kinase activity assays

The cAMP levels and PKA activity in tissue samples were measured by cAMP Enzyme Immunoassay and PKA kinase activity kit, respectively (Enzo Life Sciences, Farmingdale, NY).

Cell cultures

Bone marrow-derived macrophages (BMM), separated from femurs/tibias of C57BL/6 mice were cultured (5×10⁶/well) with 10% L929 conditioned medium for 6 days. The cell purity was 94–99% CD11b⁺. BMM were activated by LPS (10ng/ml, Sigma-Aldrich) in the presence of PACAP27, PACAP38 (10nM), or PBS control, and incubated for 24h. The H-89 (10µM) pretreatment at 1h before LPS stress was used to block cAMP-PKA pathway. Cell-free supernatants were assayed for cytokine levels by ELISA (eBioscience, San Diego, CA).

Mouse hepatocytes were isolated by *in situ* two-stage collagenase perfusion method, cultured with complete L-15 medium plus 6.25µg/ml insulin, 1µM dexamethasone, and 10% fetal bovine serum for 24h before experiments. Hepatocyte viability after isolation was 95–99%. After pretreatment with PACAP27, PACAP38 (10nM), with H-89 (10µM) or DMSO control for 1h, hepatocyte death was induced by hydrogen peroxide (5mM, Sigma-Aldrich), or TNF-α (10ng/ml, R&D Systems, Minneapolis, MN) in combination with actinomycin D (0.4µg/ml, Sigma-Aldrich) during 5h incubation period. Cells were processed for flow cytometry/caspase-3 activity, whereas supernatants were assessed for ALT/LDH levels.

Lactate dehydrogenase (LDH) release assay

Culture medium LDH activity was measured by LDH kit (Stanbio). Untreated hepatocyte lysates were used to determine total LDH activity. Cell death was expressed as LDH activity released from the treated cells as a percentage of the total LDH activity.

Flow cytometry

Hepatocytes stained with FITC-ANNEXIN V and 7-AAD (BD Biosciences, Mountain View, CA) were analyzed on a FACS-Calibur cytometer (BD Biosciences). Dead cells were identified as ANNEXIN V⁺7-AAD⁺.

Statistical analysis

All values are expressed as the mean±standard deviation (SD). Data were analyzed with an unpaired, two-tailed Student's-t test. $P<0.05$ was considered to be statistically significant.

Results

Local PACAP expression profile in liver IRI

First, we determined whether IR triggers the expression of endogenous PACAP and PACAP receptor genes in mouse livers subjected to 90min of warm ischemia, followed by reperfusion. Compared with sham controls, PACAP mRNA levels transiently dropped after the ischemia insult (0h), and increased progressively thereafter, peaking by 12–24h of reperfusion (Fig. 1a). The hepatic expression of PACAP receptors, VPAC1 and VPAC2 increased sharply at the beginning of reperfusion. While the expression of VPAC1 reduced gradually and VPAC2 dropped rapidly during the first 6h of reperfusion, both increased steadily thereafter. The expression of the PAC1 receptor increased gradually from the onset of ischemia throughout the 24h reperfusion period (Fig. 1a).

PACAP deficiency sensitizes liver to IRI

To address whether the expression of PACAP neuropeptide is essential in liver homeostasis, we assessed the impact of PACAP deficiency in our model of 90min ischemia followed by 6h of reperfusion. Indeed, PACAP KO mice showed increased susceptibility to hepatic IRI, evidenced by higher sALT levels (Fig. 1b: 31172 ± 6994 vs. 4680 ± 554 U/L; $p<0.001$) and liver histology, with more severe lobular edema, widespread hemorrhage, and congestion/hepatocellular necrosis, compared with WT controls (Fig. 1c).

Treatment with PACAP neuropeptide ameliorates liver IRI

To directly test the functional significance of PACAP, separate groups of WT mice were pretreated with PACAP neuropeptide. Unlike controls given PBS, mice conditioned with PACAP27/PACAP38 were resistant against IRI, evidenced by reduced sALT levels (Fig. 1d: $831\pm76/984\pm165$ vs. 5225 ± 630 U/L; $p<0.001$); well-preserved hepatic architecture (Fig. 1e: minimal sinusoidal congestion, no edema, vacuolization or necrosis); and decreased Suzuki's score ($p<0.001$; Supplementary Fig. 1).

PACAP depresses neutrophil/macrophage sequestration in IR-livers

MPO-based liver neutrophil activity (U/g) was depressed in mice pretreated with PACAP27/PACAP38, compared with controls (Fig. 2a: $0.46\pm0.22/0.67\pm0.06$ vs 1.56 ± 0.34 ; $p<0.01$). These correlated with the frequency of neutrophils sequestered in the livers. Their accumulation in PACAP27/PACAP38-treated mice was decreased, compared with controls (Fig. 2b: $2.3\pm1.3/3.3\pm1.3$ vs 27.8 ± 6.8 ; $p<0.001$). The parallel macrophage recruitment was also ameliorated in PACAP27/PACAP38-treated ischemic livers (Fig. 2c: $3.5\pm1.3/3.8\pm1.0$ vs 62.8 ± 3.8 ; $p<0.001$).

PACAP differentially regulates IR-induced liver cytokine/chemokine programs

To assess the immunoregulatory function of PACAP neuropeptide, we next analyzed hepatic chemokine/cytokine expression patterns. The neutrophil/monocyte-derived pro-

inflammatory chemokine (CXCL-1, CCL-2, and CXCL-10) and cytokine (TNF- α , IL-1 β , IL-6, and IFN- β) programs were markedly and uniformly suppressed in PACAP treatment groups, compared with controls (Fig. 2d,f; $p<0.001$, $p<0.01$, $p<0.05$). However, elevated IL-10 levels were noted selectively after PACAP treatment (Fig. 2e; $p<0.001$).

PACAP inhibits IR-mediated liver necrosis/apoptosis

We next screened for IR-induced hepatic oncotic necrosis and apoptosis. PACAP27/PACAP38 treatment diminished otherwise abundant hepatocellular necrosis/apoptosis, evidenced by reduced frequency of TUNEL⁺ cells (Fig. 3a, b: $2.8\pm1.0/3.0\pm1.4$ vs. 30.6 ± 4.9 [PBS]; $p<0.001$) and decreased caspase-3 activity (Fig. 3c: $5.8\pm0.8/4.6\pm0.9$ vs. 22.2 ± 1.0 [PBS]; $p<0.01$). In addition, Western blot analysis has revealed selectively increased expression of Bcl-2/Bcl-xl, yet suppressed phosphorylation of I κ B α /NF- κ B p-65 proteins after PACAP treatment, compared with PBS group (Fig. 3d).

The cAMP-PKA pathway is critical in PACAP regulation of liver IRI

Having shown that PACAP suppressed macrophage function via cAMP-PKA (17), we then asked whether PACAP may trigger cAMP-PKA signaling in our model. Indeed, we have recently found that IR itself may trigger cAMP expression (18). Interestingly, administration of PACAP27/PACAP38 neuropeptide increased cAMP levels (Fig. 4a: $1025\pm224/1085\pm233$ vs. 510 ± 88 ; $\mu\text{mol/g}$; $p<0.01$), and PKA activity (Fig. 4b: $9.9\pm0.2/10.4\pm1.5$ vs. 5.0 ± 0.2 ; ng/g ; $p<0.01$), compared with controls.

Next, we used H-89, a specific PKA inhibitor, to study as to whether cAMP-PKA activation is essential for PACAP-mediated neural immunomodulation. Strikingly, adjunctive inhibition of PKA activity not only restored but even exacerbated liver IRI in PACAP-pretreated mice, evidenced by increased sALT levels (Fig. 4c: 6115 ± 2141 [PACAP27+H-89] vs. 1165 ± 496 [PACAP27]; 6911 ± 1668 [PACAP38+H-89] vs. 2371 ± 680 [PACAP38] U/L; $p<0.001$) and hepatic histology. Livers after combined PACAP and PKA inhibition therapy were characterized by extended zonal/pan-lobular parenchyma necrosis, with widespread sinusoidal congestion and severe edema (Fig. 4d), comparable with PBS controls (Supplementary Fig. 1). Intrahepatic expression of pro-inflammatory CXCL-10, TNF- α , and IL-1 β was uniformly heightened whereas IL-10 levels concomitantly diminished after PACAP plus PKA antagonist treatment (Fig. 4e).

PACAP directly regulates macrophage TLR4 response

TLR4 activation represents the pivotal triggering step in IR-mediated liver inflammation (2). Since macrophages express three PACAP receptors (14), TLR4 regulation may contribute to the beneficial effect of PACAP in our model. First, we asked as to whether and how PACAP-triggered cAMP-PKA may affect macrophage TLR4 responses. Bone marrow-derived macrophage (BMM) cultures were stimulated with LPS in the absence or presence of PACAP; with H-89 (PKA inhibitor) or DMSO (control). Fig. 5a,b,c show that PACAP28/PACAP38 supplement depressed ($p<0.01$, $p<0.001$) otherwise enhanced LPS-induced expression (pg/ml) of TNF- α ($353.8\pm14.8/481.2\pm39.8$ vs. 959.6 ± 52.5); IL-6 ($301.2\pm59.8/565.6\pm120.0$ vs. 2188.0 ± 142.5) and IL-12p40 ($2145.9\pm99.0/2382.9\pm117.7$ vs. 5225.5 ± 80.9), but increased anti-inflammatory IL-10 (Fig. 5d: $3823.1\pm188.2/3031.5\pm93.9$ vs. 1161.3 ± 23.1 ; $p<0.001$). In contrast, H-89-facilitated PKA inhibition resulted in enhanced ($p<0.01$, $p<0.001$) TNF- α , IL-6, and IL-12p40 levels (pg/ml) in PACAP27/PACAP38 groups (Fig. 5a,b,c: TNF- α : $1074.1\pm33.8/1117.8\pm22.6$; IL-6: $1690.9\pm174.9/1986.4\pm97.6$; and IL-12p40: $4805.1\pm271.0/5347.1\pm168.1$), compared with PACAP cultures only. Moreover, IL-10 levels decreased ($p<0.001$) in BMM cultures supplemented with PACAP plus H-89 (Fig. 5d: $833.2\pm124.9/981.1\pm126.8$), compared with PACAP alone.

PACAP prevents hepatocyte death

To analyze the immunomodulatory function of cAMP-PKA signaling in hepatocytes, we designed primary hepatocyte culture systems to mimic liver IR-mediated hepatocellular damage *in vivo*. Since necrosis and apoptosis are essential in the mechanism of liver IRI, we used hydrogen peroxide (H₂O₂) to mimic *in vivo* ROS-triggered hepatocyte necrosis, or TNF- α /actinomycin D (ActD) to induce apoptosis. Native mouse hepatocytes were cultured in the presence of PACAP; with H-89 (PKA antagonist); or DMSO (control). Addition of PACAP27/PACAP38 consistently suppressed the hepatocyte death, assessed by FACS-assisted frequency (%) of Annexin V⁺7-AAD⁺ cells (Fig. 6a: H₂O₂ – 3.3 $\pm$ 2.6/3.4 $\pm$ 2.8 vs. 13.8 $\pm$ 3.6; TNF- α +ActD – 4.8 $\pm$ 2.3/3.1 $\pm$ 2.5 vs. 15.6 $\pm$ 2.5; p <0.001); diminished caspase-3 activity (pmol/min/5 $\times$ 10E4 cells) (Fig. 6b: H₂O₂ – 0.09 $\pm$ 0.07/0.09 $\pm$ 0.07 vs. 0.29 $\pm$ 0.17; TNF- α +ActD – 0.58 $\pm$ 0.13/0.58 $\pm$ 0.13 vs. 1.91 $\pm$ 0.32; p <0.001); LDH release (%) (Fig. 6c: H₂O₂ – 10.39 $\pm$ 2.29/10.36 $\pm$ 2.28 vs. 19.19 $\pm$ 5.26; TNF- α +ActD – 15.58 $\pm$ 4.23/15.54 $\pm$ 4.22 vs. 37.62 $\pm$ 9.58; p <0.01); and ALT release (%) (Fig. 6d: H₂O₂ – 10.98 $\pm$ 2.06/11.06 $\pm$ 2.03 vs. 22.58 $\pm$ 4.58; TNF- α +ActD – 13.97 $\pm$ 3.80/14.10 $\pm$ 3.75 vs. 36.36 $\pm$ 8.58; p <0.01), as compared with controls. In contrast, PKA inhibition enhanced hepatocyte death (Fig. 6a: H₂O₂ – 10.1 $\pm$ 3.1/11.2 $\pm$ 3.2; TNF- α +ActD – 13.4 $\pm$ 2.7/13.3 $\pm$ 2.8); and caspase-3 activity (Fig. 6b: H₂O₂ – 0.27 $\pm$ 0.17/0.26 $\pm$ 0.16; TNF- α +ActD – 1.85 $\pm$ 0.31/1.74 $\pm$ 0.30). In addition, PKA inhibition increased LDH (Fig. 6c: H₂O₂ – 18.63 $\pm$ 5.03/18.45 $\pm$ 5.03; TNF- α +ActD – 36.22 $\pm$ 9.24/35.88 $\pm$ 9.22); and ALT (Fig. 6 d: H₂O₂ – 21.97 $\pm$ 4.63/22.20 $\pm$ 4.57; TNF- α +ActD – 35.15 $\pm$ 8.49/35.52 $\pm$ 8.39) release in hepatocyte cultures.

Discussion

Although PACAP neuropeptide regulates macrophage cytokine programs and stimulate hepatocyte glucose production (22), its role in innate immunity-driven liver inflammation and IR-hepatocellular injury have not been explored. Here, we show that: i/ PACAP and its intrinsic receptors were induced in mouse livers subjected to warm IR; ii/ PACAP deficiency exacerbated liver damage, implying PACAP is essential for liver homeostasis; iii/ exogenous PACAP protected livers against IRI by inhibiting macrophage function and improving hepatocyte survival; iv/ PACAP-mediated regulatory/cytoprotective function was cAMP-PKA dependent.

PACAP neuropeptide may affect a diverse range of physiological functions. Indeed, PACAP-deficient mice display increased cold stress (23), decreased reproductive function (24) and altered metabolism (25). It was also shown that PACAP ablation results in higher susceptibility to renal IRI (26,27), consistent with PACAP-facilitated cytoprotection against oxidative stress in an *in vitro* primary kidney cell culture (28). However, PACAP failed to salvage hepatocellular carcinoma cell lines, perhaps due to uncertain expression of PACAP receptors on tumorized cells (28). We first found that warm ischemia and reperfusion did trigger local PACAP and all three receptor expression in the stressed liver, the levels of which were elevated between 12–24h of reperfusion (self-repair phase). This may imply the importance of PACAP neural regulation in liver's self-healing due to IRI. Then, we used PACAP KO mice to study the requirement for PACAP innervations/regulation in hepatic homeostasis. Strikingly, mice lacking PACAP neuropeptide experienced heightened liver damage, evidenced by sALT levels and histological Suzuki's grading of liver injury. We reported similarly exacerbated IRI in livers deficient of PD-1 (21) and TIM-3 (29) negative T cell co-stimulation signaling. In analogy with cytoprotection rendered by stimulating PD-1–B7-H1 pathway (21), we then asked whether administration of PACAP neuropeptide may affect liver function. Strikingly, both PACAP27 and PACAP38 diminished IR-hepatocellular damage, evidenced by decreased sALT levels and amelioration of cardinal features of liver injury, i.e., edema, vacuolization and necrosis.

In the initial IR-mediated inflammation phase, we found increased activation/recruitment of CD68⁺ macrophages, consistent with preferential pro-inflammatory chemotactic gene expression in IR-stressed livers (2–4). As PACAP therapy suppressed macrophage function (16), others have suggested that PACAP may act as an essential neural immunomodulator in autoimmune diseases (30). We observed decreased CD68⁺ macrophage infiltration and diminished activation/function, evidenced by immunohistology and decreased expression of IRI signature markers, i.e., TNF- α , IL-1 β , IL-6, CXCL-10 and CCL-2 (MCP-1). Indeed, CXCL-10, one of the key mediators in type I IFN pathway downstream of TLR4 in liver IRI (3,4), may be directly regulated by PACAP. In agreement with our *in vivo* findings, PACAP supplement diminished TLR4-mediated pro-inflammatory cytokine programs in BMM culture system.

The cAMP-PKA intracellular signaling is involved in the neural regulation by PACAP (17,31), and may modulate multiple intracellular events (32). We have identified cAMP-PKA activation as a regulator of liver IRI cascade, which halts pathological cell sequestration, prevents destructive immune reactions, and ultimately promotes parenchymal cell survival (18). It is plausible that PKA activation raises the defensive threshold to inflammatory response in IR-livers. Indeed, administration of PACAP27/PACAP38 augmented cAMP levels and enhanced PKA activity in IR-livers. Furthermore, inhibition of PKA re-created pro-inflammatory cytokine profiles in PACAP-treated BMM cultures, confirming altered liver inflammation phenotype to be responsible for local cytoprotection. Strikingly, *in vivo* PKA antagonism not only rendered otherwise IR-resistant PACAP treated hosts susceptible to the panoply of hepatic pro-inflammatory events, but also readily restored liver IRI pathology.

TLR4 activation promotes innate responses through MyD88- or TRIF-dependent pathway (33). Our previous studies indicated that signaling via TRIF-IRF3 rather than MyD88, is instrumental for downstream NF- κ B activation, local inflammation, and hepatocellular damage (2,4). We have shown that cAMP-PKA activation may directly inhibit NF- κ B by modulating p65 phosphorylation, stabilizing/elevating I κ B, as well as regulating transactivation/stability of NF- κ B complexes (18). The cAMP-PKA may also indirectly enhance CREB phosphorylation, which has higher affinity for CREB-binding protein (CBP), resulting in sequestration of p65/CBP complexes in IR-livers (18). Here, PACAP-induced cAMP-PKA activation decreased phosphorylation/proteolytic degradation of I κ B subunit, and suppressed phosphorylation of NF- κ B p65 (Fig. 7). Further, our qRT-PCR showed that PACAP inhibited downstream TLR4-NF- κ B pro-inflammatory programs, abolished TNFR/IL-1R *de novo* activation, yet augmented IL-10, all of which enhance hepatocyte survival. In agreement with *in vivo* data, we found that PKA activation diminished pro-inflammatory cytokine profile in LPS-activated BMM cultures.

Activated neutrophils generate ROS to dominate tissue damage in the second phase of liver IRI (1). Indeed, unlike in sham-controls, Ly-6G⁺ neutrophil infiltration and MPO activity increased in PBS-treated IRI. In contrast, livers in PACAP-conditioned mice were characterized by decreased neutrophil infiltration/MPO activity and depressed CXCL-1 (KC) levels, the key neutrophil chemoattractant. As neutrophil activation and target tissue sequestration can be enhanced by macrophage-derived inflammatory cytokines, PACAP can exert its regulatory function during liver IRI through cytokine/chemokine networks.

Both necrosis and apoptosis are responsible for hepatocyte damage in liver IRI (34). The death receptor activation, mitochondrial Ca²⁺ loading, and ROS promote mitochondrial permeability transition, leading to hepatocellular swelling, rupture of the plasma membrane, release of cytochrome C, ultimately resulting in ATP depletion-dependent oncotic necrosis and caspase-dependent apoptosis (1). Hepatocyte oncotic necrosis and apoptosis, which

render parenchymal cytodestruction, proceed via DNA degradation detected by TUNEL assay (34). Consistent with the essential role of PACAP in hepatic homeostasis, PACAP deficiency exacerbated hepatodestruction, increased frequency of TUNEL⁺ cells and augmented caspase-3 activity (data now shown). Conversely, PACAP treatment inhibited necrosis/apoptosis, evidenced by decreased frequency of TUNEL⁺ cells and caspase-3 activity in IR-livers. Interestingly, PACAP enhanced hepatic expression of Bcl-2/Bcl-xl, suggesting PKA activation-mediated cytoprotection by anti-necrotic/apoptotic proteins. It is plausible that neural immunomodulation prevents hepatocellular damage by modifying pro-/anti-apoptotic ratio, decreasing the release of apoptogenic factors, such as cytochrome c from mitochondria into the cytosol, maintaining mitochondria integrity, or promoting ATP generation (35).

To distinguish between necrosis and apoptosis in our *in vitro* hepatocyte cultures, we employed hydrogen peroxide (H₂O₂) to mimic *in vivo* ROS-triggered necrosis; and TNF- α to induce apoptosis. Interestingly, PACAP supplement diminished hepatocyte death, reduced caspase-3 activity, and ameliorated ALT/LDH release in both culture systems. These results, in agreement with our *in vivo* data, reinforce the immunomodulatory role of PACAP to depress NF- κ B not only in nonparenchymal but also in parenchyma cells, with resultant improvement of liver function. Furthermore, PKA inhibition exacerbated hepatocyte death, confirming this neural regulation at the hepatocyte level is cAMP-PKA dependent.

In conclusion, this study is the first to document: i/ the essential role of intrinsic PACAP neuropeptide to maintain hepatic homeostasis in liver IR-inflammation/damage, and ii/ the efficacy of exogenous PACAP to ameliorate liver IRI by depressing macrophage function in cAMP-PKA dependent manner, and to improve hepatocyte survival. Harnessing immune regulatory and cytoprotective mechanisms by neuropeptide PACAP may be essential in the maintenance of hepatic homeostasis *in vivo* by minimizing local organ damage and promoting IL-10 dependent cytoprotection. Several clinical trials suggest that PACAP38 at picomolar concentrations is safe for clinical use and has no direct effect on the circulation or regional cerebral blood flow (36, 37). As neuropeptides are currently being developed into a new therapeutic principle for chronic inflammatory lung disorders in sarcoidosis patients (38), they should also be considered as a novel therapeutic means to manage liver inflammation and IRI in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

cAMP	cyclic adenosine monophosphate
CREB	cAMP response element-binding
IRI	ischemia and reperfusion injury
LDH	lactate dehydrogenase
mAb	monoclonal antibody
MPO	myeloperoxidase

PACAP	Pituitary Adenylate Cyclase-Activating Polypeptides
PKA	protein kinase A
ROS	reactive oxygen species
sALT	serum alanine aminotransferase
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
WT	wide type

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Figure 1.

(a) Liver IRI triggers PACAP and PACAP receptor (VPAC1, VPAC2, PAC1) gene expression. Liver samples were harvested from B6 mice that were either sham-operated or subjected to 90min of partial warm ischemia, followed by various lengths of reperfusion. Livers in groups of WT, PACAP KO mice; and WT mice pretreated with PACAP27, PACAP38 or PBS were subjected to ischemia (90min). At 6h of reperfusion the hepatocellular function was analyzed by (b,d) sALT levels, and (c,e) liver histology (representative H&E staining; magnification $\times 100$ and $\times 400$) (* $p < 0.001$, $n = 10-12$ /group).

Figure 2.

Accumulation of neutrophils and macrophages in IR-livers following administration of neuropeptide PACAP (6h of reperfusion after 90min of ischemia).

(a) MPO levels; (b) Ly-6G⁺ neutrophils; (c) CD68⁺ macrophages in IR-liver lobes (magnification $\times 400$) (** $p < 0.01$, $n = 10-12$ /group).

Quantitative RT-PCR-assisted detection of cytokines/chemokines in mouse livers (6h of reperfusion after 90min of ischemia: (d) CXCL-1, CCL-2, CXCL-10; (e) IL-10; and (f) TNF- α , IL-1 β , IL-6, IFN- β). Data normalized to HPRT gene expression (** $p < 0.01$, * $p < 0.001$, $n = 4-6$ /group).

Figure 3. Necrosis/apoptosis in IR-livers (6h of reperfusion after 90min ischemia). (a) and (b) TUNEL-assisted detection of hepatic necrosis/apoptosis (dark arrows) in ischemic liver lobes (magnification $\times 400$); (c) caspase-3 activity; (d) Western blot-assisted detection of Bcl-2/Bcl-xl; p-I κ B α , and p-NF- κ B p65. (** $p < 0.01$, * $p < 0.001$, $n = 4-6$ /group).

Figure 4.

The functional significance of cAMP-PKA in PACAP neural regulation. Administration of PACAP peptides increased liver cAMP levels (a), and elevated PKA activity (b). Adjunctive use of H-89, a PKA inhibitor, restored liver injury in PACAP-pretreated groups, evidenced by (c) sALT levels (* $p < 0.001$); (d) liver histology (representative H&E; magnification $\times 100$ and $\times 400$); and (e) CXCL-10, TNF- α , IL-1 β expression (** $p < 0.05$, * $p < 0.001$).

Figure 5.

The effects of PACAP upon macrophage TLR4 activation *in vitro*. Bone marrow-derived macrophages were stimulated with LPS in the absence or presence of PACAP peptides with H-89 (PKA inhibitor), or DMSO (control). The production of (a) TNF-α, (b) IL-6, (c) IL-12p40, (d) IL-10 in culture supernatants. (**p<0.01, *p<0.001). Representative of n=3/group.

Figure 6.

The cytoprotective effects of PACAP peptides upon hepatocytes *in vitro*. Hydrogen peroxide (H₂O₂), or TNF-α+actinomycin D (ActD) were used to induce primary murine hepatocyte necrosis/apoptosis, in the absence or presence of PACAP peptides with H-89 (PKA inhibitor), or DMSO (control). (a) FACS-assisted detection of ANNEXIN V⁺7-AAD⁺ dead cells; (b) Hepatocellular caspase-3 activity; (c) LDH release; and (d) ALT levels in supernatants (**p<0.05, *p<0.01, ***p<0.001). Representative of n=3/group.

Figure 7.

A scheme of molecular mechanisms of cAMP-PKA-dependent PACAP mediated inhibition of TLR-4–NF-κB axis. PACAP binding to its receptors on macrophages (a) triggers cAMP-PKA pathway, which (b) directly prevents NF-κB translocation, and (c) stabilizes IκB inhibitor. This transcriptional machinery suppresses pro-inflammatory programs but promotes IL-10.