

**Mini Review**

## **The corticotropin releasing factor system in the liver: expression, actions and possible implications in hepatic physiology and pathology**

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### **ABSTRACT**

The corticotropin-releasing factor (CRF) system plays a crucial regulatory role in the adaptation to exogenous and endogenous stress stimuli, as well as homeostasis. Apart from the central nervous system (CNS), the members of this neuropeptide family extend their actions in the periphery, where they may affect various body systems independently, stimulating peripheral CRF receptors via vagal and/or autocrine/paracrine pathways. Here, we review all findings concerning the expression and role of the CRF system in human liver, but also in other species. Direct and indirect regulatory data are also analyzed in order to draw conclusions about possible physiological/pathophysiological implications. Although data supporting any clinical significance are still limited and further research in the field is necessary, scientific interest in the CRF system is particularly active, with multiple ongoing clinical studies evaluating the activity of CRF ligands in medical conditions involving other organs. Thus, new knowledge with therapeutic potential appears to be steadily accumulating.

**Key words:** Cancer, Corticotropin Releasing Factor, Hepatic, Liver, Neuropeptide, Receptor, Urocortin

### **INTRODUCTION**

In the mid 1950s two Nobel laureates, Dr Schally and Dr Guillemin, discovered independently the

existence of a compound with stimulatory action on adrenocorticotropin hormone (ACTH) secretion from the pituitary gland in rats and named it corticotropin-releasing factor (CRF).<sup>1,2</sup> Its structure remained unclear for almost three more decades, until Vale and his associates identified CRF as a 41-residue amino acid peptide, isolated from ovine hypothalami.<sup>3</sup> Scientists from the same research group cloned two CRF receptors, CRF<sub>1</sub><sup>4</sup> and CRF<sub>2</sub>,<sup>5</sup>

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described urocortins (additional members of the CRF family)<sup>6-9</sup> and synthesized peptide antagonists of CRF receptors; the latter substantially contributed to elucidating molecular mechanisms regulated by the CRF system.<sup>10-12</sup>

### THE CRF FAMILY: NEUROPEPTIDES AND RECEPTORS

The CRF family consists of four peptides, including CRF, urocortin 1 (Ucn 1), Ucn 2, Ucn 3 and their binding sites.<sup>13</sup> The 41 aa peptide CRF has a common primary structure among mammals, such as humans, primates, dogs and rodents<sup>14</sup> and mediates neuroendocrine and behavioral stress responses through the regulation of ACTH secretion by the pituitary gland. Ucn 1 is a 40 aa peptide that displays highly conserved primary structure among mammalian species, such as sheep, rat and mouse; it shares 45% sequence homology with CRF.<sup>15,16</sup> Ucn 1 may be traced in the pituitary, hypothalamus, the Edinger-Westphal locus and the periphery, including the gastrointestinal system, thymus, heart, spleen, kidneys and testis.<sup>16-19</sup> Ucn 2, a 38 aa peptide, and Ucn 3 are both expressed in multiple sites of the central nervous system (CNS), similarly to CRF and Ucn 1. Concurrently, Ucn 2 may be identified in the heart, adrenals and blood cells and Ucn 3 in the skin, muscles, adrenals and gut.<sup>7,9,20</sup>

The aforementioned four neuropeptides of the CRF system act through two receptors, CRF<sub>1</sub> and/or CRF<sub>2</sub>, which have been cloned from two separate genes that present 70% identity at the aa level.<sup>21</sup> Both CRF receptors belong to class B1 of the G-protein-coupled receptor superfamily<sup>5,22-24</sup> and, due to their extensive splicing, CRF<sub>1</sub> and CRF<sub>2</sub> present various isoforms. With regard to CRF<sub>1</sub>, CRF<sub>1a</sub>, primarily expressed in the pituitary and the brain, appears to be the unique isoform, which is coupled directly to adenylate cyclase, although new splicing variants are emerging and may play a critical role in CRF signaling.<sup>25-28</sup> Alternatively, CRF<sub>2</sub> has three functional splice variants (CRF<sub>2a</sub>, CRF<sub>2b</sub> and CRF<sub>2c</sub>) differing in their extracellular N-terminal domain. They are located in the CNS, skeletal muscles, heart and testis.<sup>5,13,22,29-31</sup> The CRF system receptors display different binding characteristics with their ligands. The CRF<sub>1a</sub> has high affinity to CRF and Ucn 1 and no affinity to Ucn 2 and

Ucn 3. On the contrary, the CRF<sub>2a</sub> and CRF<sub>2b</sub> display high affinity to Ucn 1, Ucn 2 and Ucn 3 and lower affinity to CRF.<sup>8,9,13,23,32,33</sup> Although Ucn 2 and Ucn 3 have been identified as selective endogenous ligands for CRF<sub>2</sub>, so far, no endogenous ligand exclusively binding to the CRF<sub>1</sub> has been identified.<sup>34</sup> Finally, CRF-BP is a 37kDa glycoprotein that was initially identified in the human plasma, dimerizing upon binding to both CRF and Ucn 1 and thus controlling the bioavailability of these peptides.<sup>35</sup>

### CRF NEUROPEPTIDES AND RECEPTORS IN THE PERIPHERY

The neuropeptides of the CRF system were initially thought to be restricted to the pituitary and brain; however, subsequent research discovered that they were widely expressed in the periphery, including multiple non-neuronal sites.<sup>36</sup> In the human periphery, CRF is expressed in adrenals (cortex and core), testis, placenta,<sup>36,37</sup> intestines, spleen, thymus, skin, pancreas, leucocytes,<sup>38,39</sup> endometrium,<sup>40</sup> ovaries<sup>41</sup> and heart.<sup>42</sup> In the rat, extra-hypothalamic CRF has been detected in the myenteric plexus and nervous fibers of the submucosal plexus in intestines and other tissues, such as testis,<sup>43</sup> ovaries,<sup>44</sup> thymus, spleen<sup>45</sup> and adrenals.<sup>46,47</sup> Furthermore, CRF has been discovered in mice,<sup>48</sup> dogs,<sup>49</sup> cows,<sup>50</sup> baboons<sup>51</sup> and monkeys.<sup>52</sup> Nonetheless, it should be noted that all studies conducted prior to the characterization of the homologue Ucn should be confirmed by selective CRF antibodies.

In addition to the brain and the pituitary, Ucn 1 is reported to be expressed in the heart, blood and lymph vessels, the reproductive organs and the gastrointestinal tract.<sup>14-18,53-55</sup> Moreover, Ucn 1 was identified in the rat autonomous nervous system of the gut at the peptide and the gene level.<sup>56,57</sup> Using RT-PCR in human tissues, Ucn 2 transcripts were detected in most sites, with higher expression levels in the heart, lung, muscle, stomach, adrenals and peripheral blood, but not in the intestine.<sup>20</sup> More recently, Ucn 2 was found in human skin,<sup>28</sup> placenta and embryonic membranes.<sup>58</sup> Ucn 3 has been identified in human CNS, heart, kidney and reproductive organs as well as the gastrointestinal system.<sup>19-21</sup>

CRF ligands and receptors were detected in the gastrointestinal tract,<sup>59,60</sup> lung, heart, spleen, testis

and connective tissue<sup>61</sup> in humans and animals. In humans, CRF<sub>2b</sub> and CRF<sub>2c</sub> were identified primarily in the brain, whereas CRF<sub>2a</sub> was expressed peripherally and centrally.<sup>22,62</sup> Ucn 1 was detected by immunostaining in the heart,<sup>63</sup> along with CRF<sub>2a</sub> and CRF<sub>1</sub>.<sup>30</sup> In contrast, in rodents, CRF<sub>1</sub> expression was reported in the submucosal and myenteric nervous plexus of the distal bowel,<sup>53,64</sup> whereas CRF<sub>2</sub> expression was predominantly detected on the luminal surface of the enteric crypts, on blood vessels of the submucosal layer<sup>53</sup> as well as on myenteric neurons.<sup>65</sup> Interestingly, CRF<sub>2a</sub> appears to be the main receptor expressed in the rat brain, whereas CRF<sub>2b</sub> is expressed in non-neuronal tissues in the brain and the periphery.<sup>66</sup>

Abundant evidence suggests the involvement of the CRF system in the inflammatory process as well as its regulatory role in the apoptotic process of macrophages,<sup>67</sup> cardiac myocytes,<sup>63,68,69</sup> adrenals,<sup>70</sup> endometrium,<sup>71</sup> colon<sup>72</sup> and kidney.<sup>73</sup> Chatzaki et al, as well as other research groups, have described the expression of Ucn and CRF receptor in both the upper and the lower human gastrointestinal tract,<sup>19,53,74,75</sup> where it appears to regulate the pathogenesis of stress-related disorders.<sup>59,76</sup>

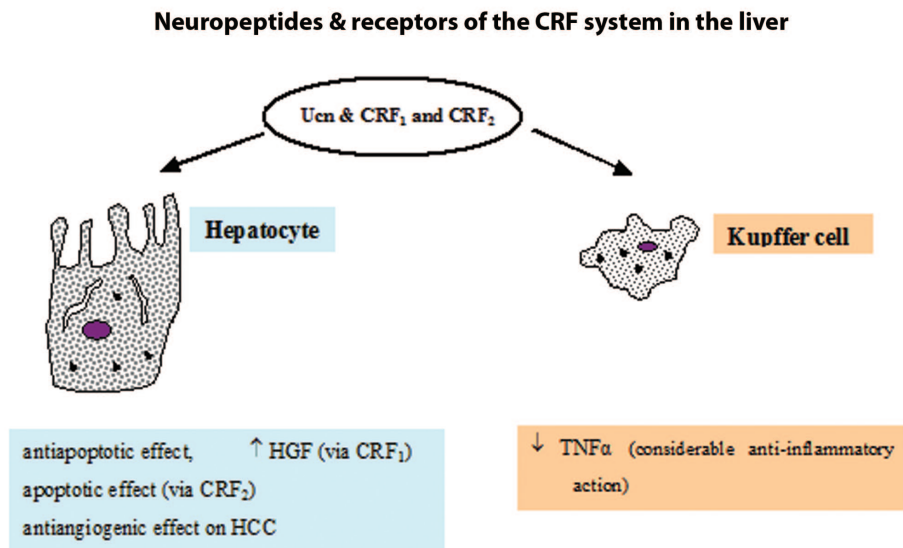
## THE CRF SYSTEM IN THE LIVER

The expression of CRF ligands and receptors in the liver has been addressed in some studies and species differences have emerged. Simopoulos et al<sup>77</sup> examined the expression of the CRF system in normal human liver tissue at gene and protein levels. Transcripts of Ucn 1, the two-receptor proteins CRF<sub>1</sub> and CRF<sub>2c</sub>, and the CRF-BP genes were detected in total RNA liver extracts by RT-PCR. The CRF and CRF<sub>2b</sub> genes were not expressed. The immunohistochemistry study showed localization of immunoreactive Ucn 1, CRF<sub>1</sub> and CRF<sub>2</sub> receptor proteins in the cytoplasm and cell membrane of hepatocytes, blood vessels and bile ducts in the portal triad. Additionally, Ucn 1 and CRF receptor expression was described in hepatic biopsies from a variety of liver pathologies, including primary or metastatic liver carcinoma and cirrhosis. A similar expression pattern of the CRF system was observed in liver cancer. Malignant cells expressed Ucn 1 and its receptors in primary hepatocarcinomas, cholangiocarcinomas and metastatic

adenocarcinomas. One cirrhotic liver biopsy was also examined and indicated Ucn 1 and CRF<sub>1</sub> expression, but not CRF<sub>2</sub>. It was concluded that the CRF system is expressed in human liver under normal and pathological conditions, with Ucn 1 being the major ligand that may act in an autocrine manner through activation of the local CRF receptors.

The expression of Ucn 1 and its receptors was also depicted in Kupffer cells (KCs), the resident macrophages that play a crucial role in the antigen-specific immune response, although in a variable way. The latter was attributed to the dissimilar degrees of maturation of KCs. The importance of the CRF system in KC activation and consequently in the hepatic immune response to noxious stimuli should be further investigated, since Ucn 1 also appeared to suppress lipopolysaccharide (LPS)-induced tumor necrosis factor alpha (TNF- $\alpha$ ) secretion by rat KCs (Figure 1).<sup>78</sup> CRF, Ucn 2 and Ucn 3 were not detected in liver cells.<sup>77</sup> Concordant with these findings, research on baboon livers showed that CRF expression is prominent at term but declines towards adulthood.<sup>79</sup>

In the study by Simopoulos et al, the presence of CRF binding protein (CRF-BP) was also discovered at the gene and protein levels in the human liver.<sup>77</sup> CRF-BP is a 37kDa glycoprotein that was firstly identified in the human plasma, dimerizing upon binding to both CRF and Ucn 1.<sup>35</sup> CRF-BP gene expression has been revealed in the brain, placenta and liver in the primates and the latter is believed to be a major source of CRF-BP secretion in the human plasma.<sup>80</sup> Nevertheless, CRF-BP was described in neither the liver nor the placenta of any of the other species examined, although it was expressed in their brain, a finding that still lacks a satisfactory explanation.<sup>81</sup> Simopoulos et al have indicated low levels of protein expression in the human liver, localized in the parenchymal hepatocytes, in addition to gene expression. It is likely that most CRF-BP synthesized in the liver is secreted into the general circulation. Through its connection with CRF and Ucn, CRF-BP may affect their bioavailability, hindering binding to CRF receptors and in that way regulating in a fast and transient way the concentration of the free peptides.<sup>82</sup> Therefore, in both neural and peripheral sites, it seems that there is a connection between CRF-BP expression and CRF or Ucn expression, as indicated in placenta<sup>81</sup>



**Figure 1.** The effects of Ucn 1 via its receptors on liver cells.<sup>76-78,101</sup>

CRF: Corticotropin Releasing Factor, HCC: HepatoCellular Carcinoma, HGF: Hepatocyte Growth Factor, TNFα: Tumour Necrosis Factor alpha, Ucn: Urocortin.

and in rat adrenals;<sup>83</sup> this may be an integral part of functional CRF-based paracrine mechanisms, since CRF-BP can bind locally secreted neuropeptides and is regulated by CRF, glucocorticoids and cytokines.

While Ucn 1 expression in the human liver was clarified,<sup>77</sup> it remains unclear in rodent livers. Rat KCs express Ucn and all its receptors, including CRF<sub>1</sub>, CRF<sub>2</sub> and the pseudoreceptor CRF-BP; consequently, KCs appear to possess a strong neuroendocrine phenotype and, reversely, the CRF system appears to influence the hepatic immunological functions.<sup>78</sup> Data from RT-PCR or Rnase protection assays suggested Ucn 1 gene expression in crude liver extracts,<sup>18,84</sup> although other investigators failed to detect this neuropeptide in rat livers.<sup>85</sup> Interestingly, differential expression patterns of the CRF system among species have also been demonstrated in other tissues and organs, such as the colon;<sup>53,74</sup> this fact may contribute to a possible explanation of the differential findings in human and rodent livers.

#### THE CRF SYSTEM IN THE LIVER: FUNCTIONAL DATA

It appears that CRF and Ucn enhances the CCl<sub>4</sub>-induced hepatic injury in rats through central receptors, while CRF antagonists alleviate this action; these

observations may have clinical prospects, because they suggest that both neuropeptides are involved in the sympathetic regulation of hepatic pathophysiology.<sup>86,87</sup> These effects were canceled through sympathectomy, although not by vagotomy of the hepatic branch; it could be concluded that they were predominantly mediated centrally.

Peripherally produced Ucn 1 within the liver may exert immediate autocrine or/and paracrine modulatory effects on the local immune response to various stimuli, promoting a different, potentially adjunct pathway in immunity. The concomitant presence of CRF<sub>1</sub> and CRF<sub>2</sub>, through which Ucn1 acts, further support the autocrine role of this neuropeptide. In fact, many reports associate CRF receptor signaling with immune and either pro- or anti-inflammatory responses in various conditions such as inflammatory bowel disease<sup>56,72</sup> and *H. pylori*-induced gastritis.<sup>19</sup> These data taken together with the expression of CRF receptors and their ligands by both KCs and hepatocytes<sup>77,78</sup> could suggest a potential role of CRF peptides as mediators of the crosstalk between liver cells and infiltrating immune cells in hepatic disorders, such as hepatitis and cancer. This hypothesis is strongly supported by the ability of CRF receptor ligands to suppress the LPS-induced production of

inflammatory TNF- $\alpha$  by rat KCs<sup>77,92</sup> and other reported actions on macrophages,<sup>67,89,90</sup> suggesting a protective, anti-inflammatory role. Ucn 1 in the hepatic parenchyma may be related to defense mechanisms activated locally to protect the liver from noxious stimuli. In fact, KCs possess important functions in the antigen-specific immune response, acting as antigen-presenting cells, interacting with hepatocytes via locally produced cytokines and/or adhesion molecule expression, and leading to a mutual influence of immunological functions. It becomes evident that Ucn's 1 role as an immune mediator in liver pathophysiology is not yet elucidated and it comprises an interesting field for further investigation.

In the rat pheochromocytoma cell line PC12, CRF favors apoptosis via CRF<sub>1</sub>, through the induction of p38-mediated Fas ligand production, possibly in a paracrine manner.<sup>91</sup> Such a Ucn/CRF-mediated paracrine regulatory loop in the liver would attract intense research interest, since it is well established that Fas ligand-mediated apoptosis is important in the pathogenesis of certain hepatic diseases, including chronic viral hepatitis and acute liver failure.<sup>92,93</sup> Further research is mandatory to investigate such involvement of the CRF system in liver pathophysiology, because it may lead to useful and important clinical discoveries.

Indeed, in order to unfold a functional biological role of these effectors in liver physiology and pathogenesis, we tested the effects of the CRF system in the hepatocellular apoptotic process, using a rat experimental model of common bile duct surgical ligation, leading to obstructive jaundice, cholestasis and apoptosis induction in the hepatic parenchyma.<sup>76</sup> Administration of selective and non-selective CRF antagonists showed that the endogenous CRF system promotes the cholestasis-induced apoptosis via CRF<sub>1</sub> activation. In contrast, CRF<sub>2</sub> seems to mediate an early and a late apoptosis-preventing phenomenon, i.e. elevated gene transcript levels of the anti-apoptotic *bcl-2* at the first postoperative day and increased rat serum hepatocyte growth factor (HGF) levels<sup>94</sup> on the third postoperative day, acting opposed to CRF<sub>1</sub>. Interestingly, no activity of CRF antagonists was observed under basal hepatic function, a finding that may imply that the CRF system plays a minimum role in the physiological turnover of the liver. Our

data point to a CRF-based apoptosis-regulating mechanism in the liver: CRF<sub>1</sub> activation resulted in apoptosis-inducing effects, whereas CRF<sub>2</sub> mediated antiapoptotic cytoprotective actions. Opposing effects of the two receptors have also been documented in other physiological pathways, such as the catecholamine secretion by adrenals.<sup>47</sup> Moreover, it has been suggested that CRF<sub>1</sub> receptor activation initiated fear and anxiety-like responses in the CNS, whereas CRF<sub>2</sub> receptor activation reestablished homeostasis by counteracting the aversive effects of CRF<sub>1</sub> receptor signaling.<sup>95</sup>

The potential regulatory activity of CRF, Ucn and their receptors in the oncogenic and carcinogenic process has been studied during the last decade with interesting results, revealing a new exciting field for CRF system-related research.<sup>96</sup> Ample experimental data have reported considerable antitumor effects of the CRF and Ucn; they appeared to inhibit the proliferation of melanoma and endometrial tumor cells, keratinocytes and human mammary cancer cells via CRF<sub>1</sub>.<sup>97-100</sup> These antitumor findings were also tested in a study showing a role of hepatic CRF receptors on tumor growth and angiogenesis.<sup>101</sup> Both *in vivo* and *in vitro* effects of Ucn1 were evaluated in human hepatoma cell lines SMMC-7721 and HepG2, human umbilical vein endothelial cells (HUVECs) and human hepatocellular carcinoma tissues. Ucn 1 inhibited the growth of hepatocellular carcinoma and reduced tumor microvessel density in nude mice. Ucn 1 administered in tumor-bearing mice inhibited the growth of established tumors *in vivo*. In addition, *in vitro* three-dimensional culture assays showed that Ucn 1 inhibited angiogenesis via CRF<sub>2</sub> activation. Finally, Ucn 1 inhibited the proliferation, promoted the apoptosis of endothelial cells and down-regulated vascular endothelial growth factor (VEGF) expression *in vivo* via CRF<sub>2</sub>. The connection of the CRF system and angiogenesis in HCC should be further studied, because it may lead to new therapeutic approaches for this lethal liver cancer. Supporting a potential clinical significance of these findings, in the study of Simopoulos et al, we described Ucn 1 and CRF receptor expression by malignant cells in primary or metastatic liver carcinoma. These observations need to be confirmed by a larger number of specimens.

## FUTURE PROSPECTS

Ucn 1 and CRF receptors in liver biopsies have recently been discovered in normal and pathological human hepatic tissue with cirrhosis and primary or metastatic carcinoma,<sup>77</sup> as well as in rat KCs.<sup>78</sup> These data suggested a receptor-mediated paracrine involvement in local inflammatory phenomena within the liver. Furthermore, an antitumor effect of Ucn in HCC and in tumors growing in rodents has been demonstrated, via activation of CRF<sub>2</sub>, which inhibited cell growth and angiogenesis,<sup>101</sup> whereas a CRF-based apoptosis-regulating mechanism was shown in

obstructive jaundice challenged liver, but not under basal conditions (Table 1).<sup>76</sup> These findings, although limited, advocate a potential regulatory role of the CRF system in the liver, regarding immunological functions, apoptotic mechanisms as well as oncogenic, antitumor and neoangiogenic processes. Taking into consideration the paramount role of the liver in the homeostasis and survival of the human body, further research addressing functionality and signaling of local receptors under basal and challenged conditions is necessary to consolidate the available knowledge concerning the CRF system impact in liver physiology and pathology and elucidate any clinical dimensions.

**Table 1.** Expression and actions of the CRF system in human and animal liver tissue. CRF: Corticotropin Releasing Factor, HCC: Hepato-Cellular Carcinoma, Ucn: Urocortin, VEGF: Vascular Endothelial Growth Factor

Study	Experimental Subject	Method	CRF Family Member	Observation-Conclusion
Simopoulos et al 2009 <sup>77</sup>	Human livers	RT-PCR, immunohistochemistry	Ucn 1, CRF <sub>1</sub> and CRF <sub>2</sub>	Ucn 1, CRF <sub>1</sub> and CRF <sub>2</sub> on hepatocytes, blood vessels and bile ducts in the portal triad.
Charalampopoulos et al 2006 <sup>78</sup>	Rats	RT-PCR	Ucn 1, CRF <sub>1</sub> and CRF <sub>2</sub>	Kupffer cells occasionally express Ucn 1, CRF <sub>1</sub> and CRF <sub>2</sub>
Paschos et al 2010 <sup>76</sup>	Rats	RT-PCR, immunohistochemistry, ELISA	CRF <sub>1</sub> and CRF <sub>2</sub>	Clolestasis-induced apoptosis is promoted by CRF <sub>1</sub> , while an antiapoptotic effect was observed for CRF <sub>2</sub>
Wang et al 2008 <sup>101</sup>	Nude mice	Three-dimensional culture assays, RT-PCR	Ucn 1, CRF <sub>1</sub> and CRF <sub>2</sub>	Ucn 1 decreased VEGF levels through CRF <sub>2</sub> in the hepatocellular carcinoma
Yokohoma et al 2001 <sup>86</sup>	Experimental acute liver injury in rats	Serum markers, liver histology	Ucn 1, CRF <sub>1</sub>	Ucn 1 exacerbated acute liver injury via CRF <sub>2</sub> in sympathetic nervous system
Nakade 2002 <sup>102</sup>	Experimental acute liver injury in rats	Serum markers, liver histology		CRF antagonists reduced liver injury

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