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Neuropeptides CRH, SP, HK-1, and Inflammatory Cytokines IL-6 and TNF Are Increased in Serum of Patients with Fibromyalgia Syndrome, Implicating Mast Cells

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ABSTRACT

Fibromyalgia syndrome (FMS) is a chronic, idiopathic condition of widespread musculoskeletal pain affecting more women than men. Even though clinical studies have provided evidence of altered central pain pathways, the lack of definitive pathogenesis or reliable objective markers has hampered development of effective treatments. Here we report that the neuropeptides corticotropin-releasing hormone (CRH), substance P (SP), and SP-structurally-related hemokinin-1 (HK-1) were significantly ($P = 0.026$, $P < 0.0001$, and $P = 0.002$, respectively) elevated (0.82 ± 0.57 ng/ml, 0.39 ± 0.18 ng/ml, and 7.98 ± 3.12 ng/ml, respectively) in the serum of patients with FMS compared with healthy controls (0.49 ± 0.26 ng/ml, 0.12 ± 0.1 ng/ml, and 5.71 ± 1.08 ng/ml, respectively). Moreover, SP and HK-1 levels were positively correlated (Pearson $r = 0.45$, $P = 0.002$) in FMS. The serum concentrations of the inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF) were also significantly ($P = 0.029$ and $P = 0.006$, respectively) higher (2.97 ± 2.35 pg/ml and 0.92 ± 0.31 pg/ml, respectively) in the FMS group compared with healthy subjects (1.79 ± 0.62 pg/ml and 0.69 ± 0.16 pg/ml, respectively). In contrast, serum IL-31 and IL-33 levels were significantly lower ($P = 0.0001$ and $P = 0.044$, respectively) in the FMS patients (849.5 ± 1005 pg/ml and 923.2 ± 1284 pg/ml, respectively) in comparison with healthy controls (1281 ± 806.4 pg/ml and 3149 ± 4073 pg/ml, respectively). FMS serum levels of neurotensin were not different from controls. We had previously shown that CRH and SP stimulate IL-6 and TNF release from mast cells (MCs). Our current results indicate that neuropeptides could stimulate MCs to secrete inflammatory cytokines that contribute importantly to the symptoms of FMS. Treatment directed at preventing the secretion or antagonizing these elevated neuroimmune markers, both centrally and peripherally, may prove to be useful in the management of FMS.

Introduction

Fibromyalgia syndrome (FMS) is a chronic medical condition characterized by widespread musculoskeletal pain, soft tissue tenderness, sleep dysfunction, stiffness, fatigue, and cognitive dysfunction (Clauw et al., 2011; Schmidt-Wilcke and Clauw, 2011; Clauw, 2014; Theoharides et al., 2015b). FMS is estimated to affect 2–8% of the adult population and is considered to be the most common cause of generalized musculoskeletal pain in women between the ages of 20 and 55 years (Branco et al., 2010). Some investigators believe that FMS belongs to a family of overlapping conditions such as chronic fatigue syndrome (CFS), irritable bowel syndrome, functional dyspepsia, myogenic temporomandibular disorder (TMD), tension headache, myofascial pain syndrome, restless leg syndrome, interstitial cystitis/bladder pain syndrome (IC/BPS), post-traumatic stress disorder, and Gulf War syndrome (Yunus, 2007; Theoharides, 2013). FMS may be triggered by Lyme disease, but in that case antibiotics do not appear to be effective (Dinerman and Steere, 1992). It should be pointed out that the pathogenesis of most of these conditions is controversial, so as new information accumulates they will likely appear to be less similar to each other than is currently perceived. Even though many patients with FMS can meet diagnostic criteria for chronic fatigue syndrome, there are distinct differences between these two groups (Abbi and Natelson, 2013).

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ABBREVIATIONS: CRH, corticotropin-releasing hormone; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; FMS, Fibromyalgia Syndrome; HK-1, hemokinin-1; IC/BPS, interstitial cystitis/bladder pain syndrome; MCs, mast cells; NK, neurokinin; NT, neurotensin; SP, substance P; TMD, myogenic temporomandibular disorder; TNF, tumor necrosis factor; VEGF, vascular enthothelial growth factor.

Diagnostic criteria for FMS have developed as a work-in-progress over the last ten years (McBeth and Mulvey, 2012; Wolfe and Walitt, 2013), but reliable diagnostic or prognostic laboratory markers would still be welcome additions. Recent research regarding the etiology of FMS has focused on central sensitization, neuroinflammation, allergens, infectious agents, irritants, chemical exposures, or physiologic stress (Russell et al., 1994, 1998; Arnold, 2010). The mediators of inflammation would include neuropeptides, cytokines, growth factors and neurotransmitters. Indeed, some neurochemical mediators have been found to be abnormal in patients with FMS (Russell and Larson, 2009; Ceko et al., 2012).

Mast cells (MCs) are involved in allergic conditions (Theoharides et al., 2015f) but can also be viewed as mediators of systemic inflammation (Galli et al., 2008b; Theoharides et al., 2012a). It has been suggested that MCs may be involved in FMS (Lucas et al., 2006; Pollack, 2015), as well as other comorbid conditions (Theoharides, 2013). The numbers of MCs were significantly increased in the papillary dermis of FMS patients (Blanco et al., 2010). Moreover, chronic urticaria, which involves MCs, has been identified in some FMS patients (Torresani et al., 2009). However, the numbers of MCs may not be as important as the degree of their activation or the specific mediators that they secrete (Theoharides et al., 2012b).

Activated MCs release numerous vasoactive, neurosensitizing, and proinflammatory mediators (Theoharides et al., 2015f) that could contribute to FMS symptoms (Theoharides et al., 2012a). In particular, MCs can release certain mediators selectively (Theoharides et al., 2007), as well as interleukin 6 (IL-6), without degranulation (Kandere-Grzybowska et al., 2003). MCs are located perivascularly in close proximity to neurons both in the skin (Paus et al., 2006) and in the diencephalon (Dimitriadou et al., 1997; Rozniecki et al., 1999). Increased levels of the neuropeptides corticotropin-releasing hormone (CRH) (Theoharides et al., 1995) and substance P (SP) may be secreted in response to physiologic or psychologic stress and could then stimulate MCs to secrete tumor necrosis factor (TNF) (Theoharides et al., 2010a).

Here we report that the neuropeptides CRH, SP, and SP-structurally-related hemokinin-1 (HK-1), as well as the cytokines IL-6 and TNF, are significantly elevated in the serum of patients with FMS compared with healthy controls.

Materials and Methods

Blood was obtained from patients (all female 28–64 years of age) with FMS. Their demographic characteristics are shown in Table 1. About 70% of these patients were medically referred to one of the authors (I.J.R.) for clinical evaluation and as potential candidates for an ongoing research study about FMS. The source of the medical referrals was an academic secondary care orthopedic clinic of the Bexar County Health Care System, affiliated with the University of Texas Health Science Center at San Antonio, to which they had been referred by their primary care physicians because of hand pain. At screening by the orthopedic physician assistants, who had been previously trained in the FMS examination by one of the authors (I.J.R.), those patients were found to meet clinical criteria for FMS and not to have convincing evidence for a compressive neuropathy (e.g., carpal tunnel syndrome). The remaining 30% were self-referred because of a physician- or presumptive self-diagnosis of FMS. All participants met 1990 American College of Rheumatology Research Classification Criteria for a diagnosis of FMS and had a pain severity of at least 40 mm, using the Pain Visual Analog Scale (1–100 mm). Potential subjects were excluded if they had a comorbid rheumatic disease, severe osteoarthritis in weight-bearing joints, an unstable or untreated endocrinopathy, any clinically significant abnormality in screening clinical laboratory tests, a severe debilitating organ failure (including heart disease, renal failure, hepatic failure), systemic cancer in the prior 6 months, uncontrolled systemic hypertension, dementia, aphasia, or other deficits of cognition or speech/language function, a history of drug or alcohol dependence, having taken long-acting opioids in the prior 3 months, lactating or pregnant women, women of child-bearing potential without a mechanical/barrier or oral anticonceptive treatment, having fainted within the last 6 months, or having received an investigational drug or device within 30 days prior to starting the study. The clinical protocol of this research study was approved by the University of Texas Health Science Center in San Antonio Institutional Review Board (IRB #901-5003-203). Patients signed informed consent to participate in the study before any research-related procedures were initiated. Serum was separated from other blood components using standard serum sample tube methodology. The samples were number coded and no personal identifiers or protected health information accompanied the samples. Within 30 minutes of collection, the serum was aliquoted and frozen at –70°C until analyzed.

Serum was also obtained from healthy subjects (5 male and 15 female, 25–65 years of age) who had no history of musculoskeletal pain or any evidence of inflammation. The normal controls were not related to any of the FMS patient group and were recruited from the Boston area or purchased from BBI Solutions (Cardiff, UK). Serum samples were labeled only with a code number and the age and gender of the subjects. All control blood samples were processed immediately after phlebotomy and the serum was stored in –80°C until it was used for analysis.

The numbers of patients included in the various measurements varied because the amounts of serum available were not consistent for all samples. The levels of SP were measured first and as subsequent measurements were made, the amounts of serum remaining decreased progressively until quantities were not sufficient for some additional tests. This was true for both the FMS and normal control serum samples.

Extraction of Serum Peptides. The extraction of serum peptides was performed using a SEP-COLUMN containing 200 mg of C18 (cat. no. RK-SEPCOL-1), Buffer A (cat. no. RK-BA-1), and Buffer B (cat. no. RK-BB-1) (Phoenix Pharmaceuticals, Inc., Burlingame, CA). A combination of a centrifugal concentrator (Savant SpeedVac SVC 100H) and a lyophilizer (Edwards K4 Modulyo Freeze Dryer, West Sussex, England) was used for drying the samples after extraction. First, SpeedVac was used to dry the samples for approximately 15 minutes to remove the organic layer. The remaining sample was snap-frozen in liquid nitrogen and freeze-dried overnight using a lyophilizer. The dried extracts were then reconstituted with 1× assay buffer and phosphate-buffered saline for measurement of CRH and neurotensin (NT) in human serum samples.

Assessment of Markers Levels in Serum. Human serum CRH, SP, and NT levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Phoenix Pharmaceuticals, Inc.) according to the manufacturer's protocol. HK-1 levels were measured using commercially available ELISA kits from Peninsula Laboratories International, Inc. (San Carlos, CA). Human serum IL-31, IL-6, IL-33, and TNF levels were determined using commercially available ELISA kits from R&D Systems (Minneapolis, MN).

Statistical Analysis. All data were validated and inspected for outliers. The results are presented as scattergrams, with symbols representing individual data points and the horizontal lines representing the mean for each group. Normality of distribution was checked with the Shapiro-Wilk test. Comparisons between the FMS group and the healthy controls were performed using the nonparametric Mann-Whitney *U* test or the unpaired *t* test. Pearson correlation analysis was performed between SP and HK-1 serum levels in

TABLE 1
Demographic characteristics of the human subject participants

Characteristics	Fibromyalgia Syndrome (n = 84)	Healthy Controls (n = 20)
Gender (% female)	100%	75%
Age range (mean years)	28-64 (47.94 ± 11.18)	25-65 (38.15 ± 10.62)
Ethnicity	90% Caucasian	100% Caucasian
Pain severity (mean PVAS)	67.7 mm	Not applicable

PVAS, Pain Visual Analog Scale.

FMS patients. A result was considered significant at a P value < 0.05. The analysis was performed by using the GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA).

Results

The demographic characteristics of the subjects included in this study are presented in Table 1. The FMS patients consisted of 84 females with a mean age of 47.9 ± 11.2 years. Ninety percent were Caucasian (35% European Caucasian, 55% Hispanic Caucasian). The average educational achievement was 13.6 years. Forty percent were gainfully employed. The healthy control group consisted of 15 females and 5 males mean age of 38.2 ± 10.6 years. One hundred percent were Caucasian. The important differences between FMS and healthy control groups were related to diagnosis of a painful multimodal medical condition in the FMS group that was not present in the healthy control group.

Serum levels of the neuropeptides CRH and SP were significantly ($P = 0.026$ and $P < 0.0001$, respectively) elevated (0.82 ± 0.57 ng/ml and 0.39 ± 0.18 ng/ml, respectively) in patients with FMS compared with healthy controls (0.49 ± 0.26 ng/ml and 0.12 ± 0.1 ng/ml, respectively) (Fig. 1, A and B). However, there was no correlation between CRH and SP serum levels.

The serum levels of HK-1 were increased ($P = 0.002$) in FMS patients (7.98 ± 3.12 ng/ml) compared with normal controls (5.71 ± 1.08 ng/ml) (Fig. 2A). Moreover, there was a significant correlation between serum levels of HK-1 and SP ($r = 0.45$, $P = 0.002$) but not CRH (Fig. 2B).

There was no statistical difference in serum levels of NT between FMS patients and healthy controls (data not shown).

The mean serum levels of the cytokines IL-6 and TNF were significantly ($P = 0.029$ and $P = 0.006$, respectively) increased (2.97 ± 2.35 pg/ml and 0.92 ± 0.31 pg/ml, respectively) in patients with FMS compared with healthy controls (1.79 ± 0.62 pg/ml and 0.69 ± 0.16 pg/ml, respectively; Fig. 3, A and B).

Serum IL-31 and IL-33 levels were significantly ($P = 0.0001$ and $P = 0.044$, respectively) lower (923.2 ± 1284 pg/ml and 849.5 ± 1005 pg/ml, respectively) in patients with FMS compared with the healthy controls (3149 ± 4073 pg/ml and 1281 ± 806.4 pg/ml, respectively) (Fig. 4, A and B).

Discussion

The demographic characteristics of the FMS and control subjects were comparable (Table 1), but it is important to point out that the FMS group was characterized only by painful multimodal symptoms not present in the healthy control group. Hence, the differences in the biomarkers reported here are clinically significant. In particular, our study shows that the neuropeptides CRH, SP, and the SP-related HK-1 are significantly elevated in the serum of FMS patients in comparison with controls. This is the first time to our knowledge that CRH and HK-1 are shown to be increased in the serum of FMS patients.

The neuroimmune findings reported here fit in a "unified" model proposed earlier but on the basis of cerebrospinal fluid (CSF) measurements (Russell and Larson, 2009). One study showed that CRH was elevated in CSF of FMS patients and was associated with pain but not fatigue symptoms (McLean et al., 2006). The increased serum CRH reported in the current study may indicate some level of physical or psychologic stress that may exacerbate FMS symptoms (Geenen et al., 2002; Fischer et al., 2016). CRH is typically secreted from the hypothalamus under the influence of stress and activates the hypothalamic-pituitary-adrenal axis but has also been shown to induce inflammation (Chrousos, 1995). CRH-positive nerve endings have been localized in the median eminence of the hypothalamus, where MCs are most plentiful (Rozniecki et al., 1999). CRH can also be released from MCs (Kempuraj et al., 2004), other immune cells (Karalis et al., 1997), and skin cells (Donelan et al., 2006b). In fact, human MCs express mRNA and protein for CRH receptor 1, activation of which induces selective release of vascular endothelial growth factor

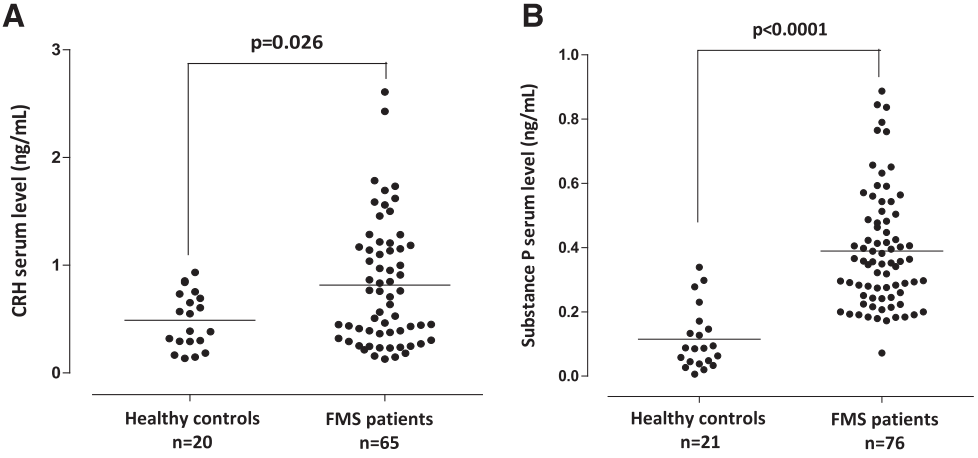

Fig. 1. (A, B) Comparison of serum CRH and SP levels in healthy controls and FMS patients. Symbols represent individual data points, and the horizontal line represents the mean for each group.

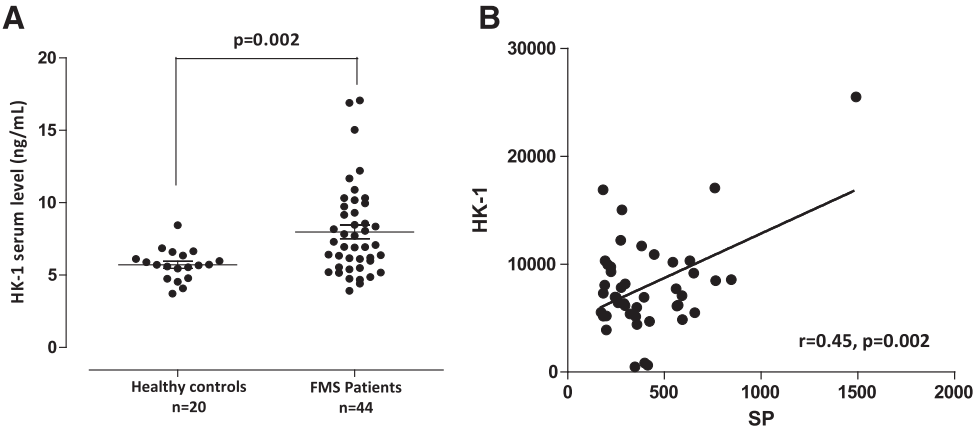

Fig. 2. (A) Comparison of serum HK-1 levels in healthy controls and FMS patients. Symbols represent individual data points, and the horizontal line represents the mean for each group. (B) Correlation between serum HK-1 and SP levels.

(VEGF) (Cao et al., 2005), leading to increased vascular permeability (Lytinas et al., 2003), disruption of the blood-brain barrier (Theoharides and Konstantinidou, 2007) through brain MC activation (Esposito et al., 2002), and potential entry of toxins and other environmental triggers into the brain.

Our present findings support previous reports of elevated CSF concentrations of SP in FMS patients (Russell et al., 1994). Substance P is an eleven amino acid peptide originally isolated and characterized from the rat brain (Leeman and Ferguson, 2000) and has been involved in the pathogenesis of inflammation (O'Connor et al., 2004; Saraceno et al., 2006; Douglas and Leeman, 2011; Munoz and Covenas, 2014; Steinhoff et al., 2014; Richard et al., 2015). The neurokinin-1 (NK-1) receptor for SP has been implicated in the pathophysiology of pain, but blockade of that receptor does not control the pain of FMS (Greenwood-Van Meerveld et al., 2014; Russell, 2002). Substance P can stimulate a number of immune cells, especially MCs (Fewtrell et al., 1982), to release TNF (Ansel et al., 1993; Okayama et al., 1998; Okabe et al., 2000; Theoharides et al., 2010b, 2012a). Moreover, SP can induce MCs to express CRH receptor 1 leading to an augmentation of the physiologic stress response (Asadi et al., 2012). It should be pointed out that depending on when CRH is released and where in the brain, it may not necessarily activate the hypothalamic-pituitary-adrenal axis and lead to increased serum cortisol. This may be the reason why morning serum cortisol is not different between FMS patients and controls, but evening cortisol is actually increased in FMS patients (Fatima et al., 2013).

It was recently reported that MCs can secrete HK-1, which augments their allergic stimulation (Sumpter et al., 2015). HK-1 is also expressed by other immune cells (Klassert et al., 2008). Both SP and HK-1 also help generate Th-17 by inducing IL-1β and TNF in monocytes (Cunin et al., 2011). HK-1 was shown to contract the human bronchi through activation of both NK-1 and NK-2 receptors (Grassin-Delyle et al., 2010).

Even though the effect of CRH on the skin was augmented by NT (Donelan et al., 2006a), NT was not increased in the serum of FMS patients. However, no one to date has studied the effect of CRH on MC stimulation by SP.

Our data has shown that the mean serum levels of the inflammatory cytokine IL-6 are significantly elevated in FMS. There is increasing evidence that changes in these inflammatory cytokine levels may influence the severity of symptoms in FMS patients (Mendieta et al., 2016; Guttenbrunner et al., 2011; Nugraha et al., 2013). In agreement with our findings, other studies reported that IL-6 was increased in the serum of FMS patients (Hernandez et al., 2010; Ross et al., 2010; Behm et al., 2012) and that its levels correlated with FMS severity (Uceyler et al., 2011) (Table 2). Nevertheless, some papers reported no difference in serum IL-6 levels between FMS patients and controls (Wallace et al., 2001; Gur et al., 2002; Kim et al., 2010). It should be noted, however, that the serum levels in the control subjects in these studies were unusually high (e.g., 5.46 $\pm$ 1.38 pg/ml; Gur et al., 2002) compared with

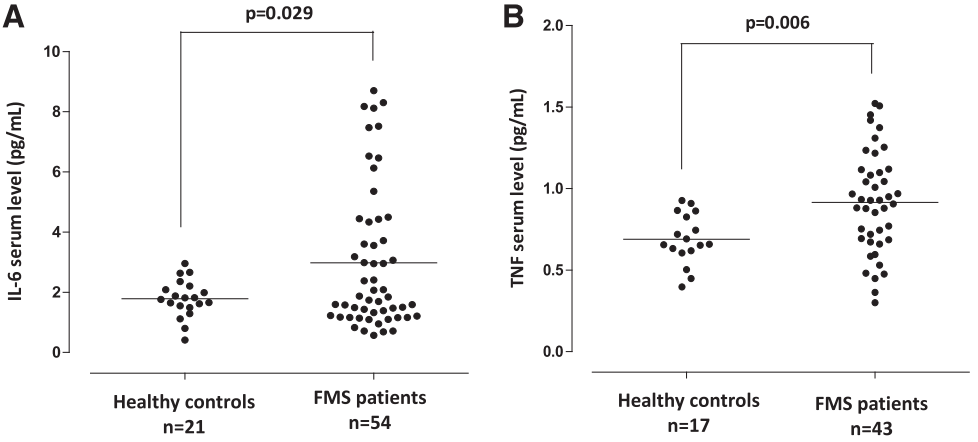

Fig. 3. (A, B) Comparison of serum IL-6 and TNF levels in healthy controls and FMS patients. Symbols represent individual data points, and the horizontal line represents the mean for each group.

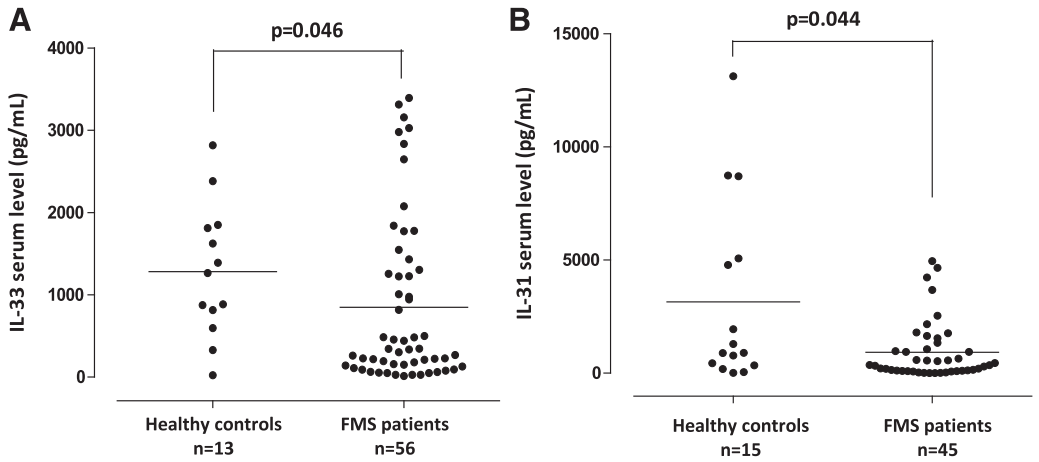

Fig. 4. (A, B) Comparison of serum IL-33 and IL-31 levels in healthy controls and FMS patients. Symbols represent individual data points, and the horizontal line represents the mean for each group.

our controls (0.92 ± 0.31 pg/ml) or those reported by Hernandez et al. (2010; 0.92 ± 0.32 pg/ml).

The increased IL-6 levels may derive from MCs because acute-restraint physiologic stress of mice led to increased serum IL-6, which was entirely MC-dependent (Geiss et al., 2011). Moreover, increased serum IL-6 was associated with bone pain in patients with mastocytosis (Theoharides et al., 2015f), who often present with comorbid FMS (Jennings et al., 2014; Theoharides et al., 2015f). It is important to note that patients with mastocytosis experience diffuse musculoskeletal pain (Delsignore et al., 1996) and are often diagnosed with FMS (Theoharides et al., 2015c).

In the present study, we also found significantly increased serum TNF levels in patients with FMS in comparison with healthy controls. One previous study (using ELISA) also reported increased plasma TNF in FMS patients (Bazzichi et al., 2007a), but other studies apparently found no difference in TNF serum levels between FMS patients and controls (Wallace et al., 2001; Garcia-Campayo et al., 2008; Ross et al., 2010). Surprisingly, another study reported decreased serum TNF levels in FMS patients compared with controls, but the levels in both patients (20.42 ± 7.24 pg/ml) and controls (35.73 ± 0.72 pg/ml) were 20 times or more higher (Hernandez et al., 2010) than the levels we report (1.79 ± 0.62 pg/ml and 0.69 ± 0.16 pg/ml, respectively).

Several studies have reported elevated levels of the proinflammatory chemokine IL-8 (CXCL8) in both serum (Wallace et al., 2001; Gur et al., 2002; Ross et al., 2010; Bote et al., 2012; Kadetoff et al., 2012; Rodriguez-Pinto et al., 2014) and CSF (Kadetoff et al., 2012) of patients with FMS (Table 2). Interestingly, one study reported that moderate (45 minutes cycling) exercise *decreased* the serum concentration of IL-8 in FMS patients, but *increased* it in healthy controls; there was also a decrease in the release of IL-6 and TNF from stimulated peripheral monocytes in FMS patients compared with healthy women (Bote et al., 2013). These findings may help to explain the apparent beneficial effect of mild exercise in FMS.

One study showed a strong correlation between increased plasma levels of IL-17A and TNF in patients with FMS

TABLE 2
Reported cytokine/chemokine levels in FMS

Cytokines	Assays	N^a	Results	References
IL-6, TNF-α (Serum)	ELISA	54:21 (IL-6), 43:17 (TNF-α)	IL-6, TNF-α ↑	Present results
IL-4, IL-6, IL-8, TNF-α (Serum)	Bio-Plex cytokine assay	20:80	IL-8, TNF-α ↑ IL-6 ↓ IL-4, IL-10 →	Wang et al. (2008)
IL-6, IL-8, TNF-α (Serum)	Luminex	56:32	IL-8 ↓ IL-6, TNF-α → IL-6, IL-8, TNF-α ↓ in older FM patients	Garcia-Lozano et al. (2008)
IL-1, IL-6, IL-8 (Serum)	ELISA	81:32	IL-8 ↑ IL-1, IL-6 →	Gur et al. (2002)
IL-8 (Serum)	ELISA	20:20	IL-8 ↑	Bote et al. (2012)
IL-1α, IL-1β, IL-6, IL-8, TNF-α (Serum)	Luminex	7:12 (Nonresponders[b])	IL-6, IL-8↑	Ross et al. (2010)
IL-1β, IL-6, IL-8 (Serum)	ELISA	27:29	IL-8 ↑ IL-1β, IL-6 →	Kim et al. (2010)
IL-1β, IL-6, TNF-α (Serum)	ELISA	64:25	IL-6 ↑ IL-1β, TNF-α ↓	Hernandez et al. (2010)
IL-6, IL-8 (Serum)	ELISA	105:61	IL-8, IL-6 →	Xiao et al. (2013)
IL-1β IL-6, IL-8, TNF-α (Serum)	ELISA	56:36	IL-8 ↑ IL-1β, IL-6, TNF-α →	Wallace et al. (2001)
IL-1β, IL-5, IL-6, IL-8, TNF-α (Serum)	ELISA	15:15	IL-8 ↑ IL-1β →	Kadetoff et al. (2012)
IL-1, IL-6, IL-8, TNF-α (Plasma)	ELISA	80:45	IL-8, TNF-α ↑	Bazzichi et al. (2007b)
IL-6, IL-8 (Plasma)	Luminex	39:47 (IL-6), 62:43 (IL-8)	IL-6, IL-8 ↓	Behm et al. (2012)

[a]Number of FMS patients/number of healthy controls.
[b]Nonresponders did not increase growth hormone levels after exercise.

(Pernambuco et al., 2013). CSF and serum IL-17 also positively correlated with measures of subjective pain among FMS patients (Meng et al., 2013), and with depression and anxiety in patients with rheumatoid arthritis (Liu et al., 2012), symptoms also reported by 30–40% of patients with FMS. TNF and IL-17 seem to act together in perpetuating the inflammatory process (Romero-Sanchez et al., 2011; Griffin et al., 2012). MC-derived IL-6 and tumor growth factor (TGF)β induce the development of Th-17 cells through dendritic cell maturation (Dudeck et al., 2011). Moreover, MCs can secrete IL-17 themselves (Kenna and Brown, 2013), as shown both in arthritic joints (Hueber et al., 2010) and in psoriatic lesions (Lin et al., 2011).

We also measured IL-31 because it has been involved in allergies (Rabenhorst and Hartmann, 2014) and IL-33 because it is involved in autoimmunity and inflammation (Theoharides et al., 2015a). The serum levels of IL-31 and IL-33 were significantly lower in the FMS group compared with healthy controls.

It is obvious from the previous publications discussed, some of which have been reviewed before (Rodriguez-Pinto et al., 2014) and are summarized in Table 2, that blood levels of cytokines vary considerably. A number of variables could influence the levels of these blood biomarkers: 1) different assay methods, 2) pre- or post-menopausal female subjects, 3) differences in Body Mass Index (BMI), 4) serum or plasma levels may not correspond to or accurately reflect tissue or circulating blood cell cytokine expression or release, 5) may be affected by concurrent medications, and 6) serum cytokine levels may vary if obtained at different times of the day. For instance, it was recently reported that MCs release their mediators in a circadian mode (Nakamura et al., 2014). Multiple cytokine testing, rather than single measurement, especially at different times of the day, could be achieved by measuring them in 24-hour urine as is done with MC mediator metabolites (Divekar and Butterfield, 2015). In addition, measurements early and late in the course of FMS might prove to be useful. Finally, measuring gene expression of cytokines in relevant tissues may be more accurate than blood levels.

We recently reviewed the pathophysiology of FMS (Theoharides et al., 2015e) and the relative lack of reliable biomarkers or effective treatments. We now show that the neuropeptides CRH, SP, and HK-1 are significantly elevated in the serum of FMS patients. These molecules could be released centrally and may cause focal inflammation leading to MC activation and pain (Heron and Dubayle, 2013). Peripheral inflammation may still have central effects (Lampa et al., 2012).

MCs and their mediators have been implicated in neuropsychiatric diseases (Theoharides et al., 2004), IC/BPS (Sant et al., 2007), and irritable bowel syndrome (Theoharides et al., 2012b). Increased number of activated MCs was also reported in skin biopsies of FMS patients (Blanco et al., 2010). MCs are located perivascularly close to neurons, especially in the leptomeninges (Polyzoidis et al., 2015), thalamus, and hypothalamus (Pang et al., 1996). We hypothesize that environmental, immune, and infectious triggers stimulate MCs, leading to secretion of inflammatory mediators (Fig. 1). Stimulated MCs secrete vasoactive and proinflammatory mediators, such as the preformed heparin, histamine, serotonin, proteases (e.g., tryptase), and TNF, as well as the de novo synthesized leukotrienes, prostaglandins, and cytokines (IL-1, IL-6, IL-8, TNF). At least histamine, prostaglandin D_2 (PDG_2), and tryptase are known to stimulate sensory nerves and elicit pain (Dai et al., 2004; Chatterjea and Martinov, 2015). In addition, histamine, PGD_2, IL-6, TNF, and tryptase could also stimulate microglia, as well as CRH release from the hypothalamus (Turnbull and Rivier, 1999). MC-microglia interactions are implicated in the pathogenesis of neuroinflammation (Skaper et al., 2012). In fact, CRH and microglia have been involved in the pathogenesis of mental disorders (Kritas et al., 2014).

Brain cells activated by these mediators or by CRH could then release HK-1 and SP, further stimulating MCs. Brain MCs were reported to synthesize and release TNF (Cocchiara et al., 1999). It is particularly important that MCs are the only immune cells that store *preformed* TNF, allowing rapid secretion (Zhang et al., 2011), and that superactivate T cells (Nakae et al., 2006; Kempuraj et al., 2008).

MCs are derived from bone marrow progenitors and mature in tissues depending on microenvironmental conditions (Galli, 1990). MCs are critical for the development of allergic reactions (Theoharides et al., 2015f) but are also implicated in immunity (Galli et al., 2008a) and inflammation (Theoharides et al., 2012a, 2015d). We have shown that certain natural flavonoids (Middleton et al., 2000), such as luteolin and quercetin (a combination of which are found in the dietary supplement FibroProtek), can inhibit IL-6 (Kandere-Grzybowska et al., 2006) and TNF (Kempuraj et al., 2005; Weng et al., 2015) release from stimulated MCs. Luteolin and quercetin could, therefore, be useful in FMS (Fig. 5). It is interesting that vitamin D inhibits MCs (Baroni et al., 2007) and has been reported to have a regulatory function in inflammatory diseases (Calton et al., 2015; Toniato et al., 2015).

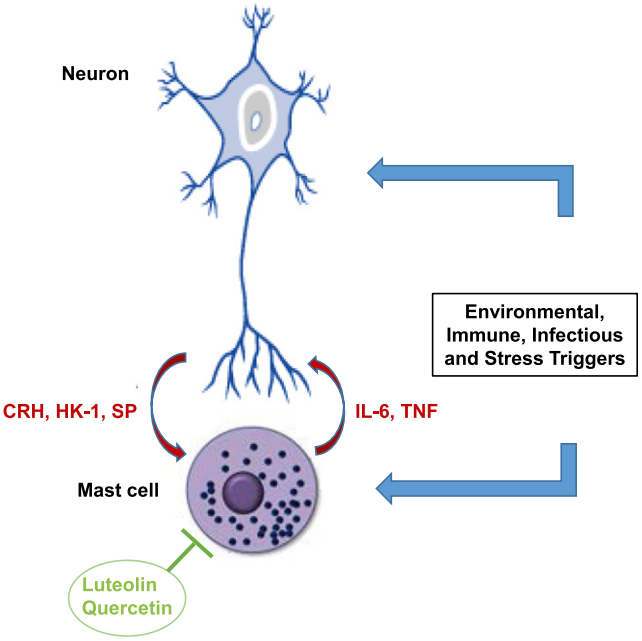

Fig. 5. Diagrammatic representation of the proposed interactions among neuropeptides, mast cells, inflammatory cytokines, neurons, and FMS pathogenesis. Environmental, immune, and infectious triggers activate MCs leading to secretion of inflammatory mediators such as IL-6 and TNF, which could further stimulate nerves to release CRH, HK-1, and SP, further stimulating MCs. Luteolin and quercetin could be of help by blocking MC stimulation and/or release of inflammatory mediators.

Conclusion

Our findings of significantly elevated serum levels of the neuropeptides CRH, SP, and SP-structurally-related hemokinin-1 (HK-1), as well as the cytokines IL-6 and TNF, in FMS patients compared with healthy controls may prove to be diagnostically useful. Preventing the secretion of CRH, SP, and/or HK-1, and their ability to stimulate release of IL-6 and TNF from MCs, microglia, or other immune cells, may constitute a potential new treatment approach for FMS.

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Authorship Contributions

Participated in research design: Tsilioni, Theoharides, Russell.

Conducted experiments: Tsilioni.

Performed data analysis: Tsilioni.

Wrote or contributed to the writing of the manuscript: Theoharides, Tsilioni, Stewart, Gleason, Russell.

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