Up-regulation of cutaneous α_1-adrenoceptors in complex regional pain syndrome type I

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Abstract

Background. In a small radioligand-binding study of cutaneous α_1-adrenoceptors in complex regional pain syndrome (CRPS), signal intensity was greater in the CRPS-affected limb than in controls. However, it was not possible to localize heightened expression of α_1-adrenoceptors to nerves, sweat glands, blood vessels or keratinocytes using this technique.

Methods. To explore this in the present study, skin biopsies were obtained from 31 patients with CRPS type I and 23 healthy controls of similar age and sex distribution. Expression of α_1-adrenoceptors on keratinocytes, and on dermal blood vessels, sweat glands and nerves was assessed using immunohistochemistry.

Results. α_1-adrenoceptors were expressed more strongly in dermal nerve bundles and the epidermis both on the affected and contralateral unaffected side in patients than in controls ($p<0.05$). However, expression of α_1-adrenoceptors in sweat glands and blood vessels was similar in patients and controls. α_1-adrenoceptor staining intensity in the CRPS-affected epidermis was associated with pain intensity ($p<0.05$), but a similar trend for nerve bundles did not achieve statistical significance.

Discussion. Epidermal cells influence nociception by releasing ligands that act on sensory nerve fibres. Moreover, an increased expression of α_1-adrenoceptors on nociceptive afferents has been shown to aggravate neuropathic pain. Thus, the heightened expression of α_1-adrenoceptors in dermal nerves and epidermal cells might augment pain and neuroinflammatory disturbances after tissue injury in patients with CRPS type I.

Key words: complex regional pain syndrome; α_1-adrenoceptors; immunohistochemistry; epidermis; dermal nerves

Introduction

Complex regional pain syndrome (CRPS) is an intriguing condition with many paradoxes (1, 2). Early descriptions of soldiers with traumatic limb injuries included signs of autonomic disturbance such as excessive sweating, oedema, temperature asymmetry and altered skin blood flow (3). During World War I and later conflicts, successful treatment of CRPS type II (causalgia) with surgical sympathectomy furthered the concept of sympathetic nervous system involvement (4, 5). Similar disturbances of autonomic function were also seen after non-combat injuries, and the term "reflex sympathetic dystrophy" (CRPS type I) was coined (6). However, sympatholytic interventions provided pain relief in only a minority of cases (7, 8). The subgroup that did respond to sympatholysis was deemed to have "sympathetically maintained" pain but the mechanism by which this could occur was unclear. In this subgroup, sympathetic blockade reduced pain and mechanical hyperalgesia that could be rekindled by the application of noradrenaline and other adrenergic agonists (9-11), suggesting that pain might be driven by excessive efferent sympathetic activity. However, this became less attractive as a pathophysiological mechanism for the development of CRPS when evidence to the contrary appeared. Specifically, concentrations of noradrenaline and its metabolite 3,4-dihydroxyphenylethyleneglycol were found to be *lower* in the affected than contralateral unaffected limb in patients with CRPS, indicating that sympathetic outflow might be compromised (12, 13). Afferent sensory-sympathetic coupling has been suggested as a mechanism for the development of sympathetically maintained pain (14, 15) but, again, evidence for this is sparse (16).

Instead, α-adrenoceptor supersensitivity might, in part, explain some of the clinical findings in CRPS (17, 18). A search for evidence of up-regulation of cutaneous adrenoceptors (α_1-ARs) led us to study their density in skin biopsies taken from normal

individuals and in patients suffering from CRPS using a selective radioligand (19). The findings indicated an increase in receptor density in CRPS-affected skin; however, a more refined technique was required to determine the distribution of α_1-ARs in skin organelles, keratinocytes and especially nerve fibres. In a subsequent animal study, immunohistochemistry was used to determine the expression of α_1-AR on primary nociceptive afferents that innervated the healthy skin of adult Wistar rats. α_1-ARs were found to co-localise with nociceptive markers in cutaneous nerve fibres, providing a possible means of excitation of these fibres by adrenergic agonists (20). We reported recently that α_1-ARs were expressed more strongly on keratinocytes and nociceptive afferents in the ipsilateral than contralateral plantar hind paw of rats after chronic constriction injury of the sciatic nerve (a model of CRPS type II) (21). Similarly, in the partial sciatic nerve ligation model, α_1-ARs were expressed more strongly on nociceptive afferents in the sciatic nerve trunk and distal skin 4 and 28 days after injury than after sham surgery (22). Furthermore, α_1-AR expression was greater on dermal nerve fibres and keratinocytes in the affected than contralateral unaffected limb of patients who had developed CRPS type II after peripheral nerve trauma.

The purpose of the current study was to investigate the expression of α_1-ARs in the skin of healthy volunteers and patients suffering from CRPS without signs of major nerve trauma (CRPS type I). As symptoms of CRPS type I are similar to those of CRPS type II, we hypothesized that these patients would have greater expression of cutaneous α_1-ARs than healthy volunteers and, in particular, increased numbers in specific structures such as neurons, keratinocytes, blood vessels and sweat glands. Up-regulation of α_1-ARs on these cells would provide evidence in support of an involvement of α_1-ARs in the pathophysiology of CRPS type I (14, 23).

Methods

Participants

Skin samples were obtained from eight men and 23 women aged between 20 and 68 years (mean age ± S.D. 41 ± 12 years) with CRPS type I in an upper or lower limb, and from 10 men and 13 women aged between 20 and 68 years (mean age 34 ± 16 years) from the university population who were free of pain. Each participant provided written informed consent for the procedures, which were approved by the institutional ethics committee.

Procedures

Each patient was reviewed by a pain physician to determine whether clinical criteria for CRPS type I were met. The syndrome began after a limb fracture (seven cases) or soft-tissue injury without obvious major nerve trunk involvement (22 cases), or started after no identifiable cause (two cases). In each case, pain and other symptoms spread beyond the injured area, sometimes to another limb, but involved the contralateral side in only three cases (one with reduction in sensation in the contralateral thigh, and another two with mild contralateral symptoms and pain). Patients with prominent bilateral symptoms were excluded. Patients provided a rating of mean pain intensity over the two weeks prior to examination on a visual analogue scale. They were also questioned about present or past sensory and autonomic disturbances in the affected limb, movement deficits and trophic changes in the hair, nails and skin. During the examination the following signs were graded as absent, mild or severe: allodynia to briskly tapping the limb with a finger and sensory blunting to painfully scratching the skin with the sharp end of a paperclip; swelling; and motor disturbances such as decreased range of movement, weakness and tremor. In addition, trophic signs and autonomic disturbances were noted.

CRPS was considered to be present when reports of sensory, and/or autonomic and/or motor disturbances were substantiated in the clinical examination, as specified in the Budapest clinical diagnostic criteria for CRPS type I (24).

Skin samples were obtained under sterile conditions from a site of mechanical or thermal hyperalgesia (generally the dorso-lateral aspect of the affected hand or foot) using a 3 mm diameter skin biopsy punch under local anaesthesia. Samples from a mirror image pain-free site on the contralateral side of the body were also obtained. In controls, skin samples were obtained from the dorso-lateral aspect of one hand or the ventral forearm. Healing was uneventful in each case.

Tissue preparation and immunohistochemistry

Skin samples were fixed in Zamboni's solution (5% formalin and 1% picric acid (v/v) in 0.9% saline) for 4 h at 4 °C. Following fixation, tissues were washed in 0.1 M phosphate buffer (PB; pH 7.4), incubated in 50% ethanol for 30 min followed by a further two rinses in PB and embedded in paraffin for storage. Skin biopsies were later sectioned at 10 μm and collected onto StarFrost® silane coated slides (ProSciTech, Queensland, Australia) and deparaffinised through xylene and a descending series of ethanol washes to 0.1 M phosphate buffered saline (PBS). Tissue sections were treated with a 1 mg/mL solution of porcine trypsin (Sigma-Aldrich, St Louis, MO, USA) for 20 min and rinsed in two changes of PBS for 2 min. For all experiments, normal control skin and tissue sections from the contralateral and ipsilateral limbs of patients with CRPS were processed simultaneously using antibodies as detailed in Table 1. In addition, two consecutive sections from the same biopsy were labelled concurrently on the same slide to check the reproducibility of staining.

To localise α_1-AR expression on blood vessels and the secretory coil of sweat glands, sections were double labelled with antibodies against α_1-AR and smooth muscle

actin (SMA). Sections were treated with 0.2% Triton X-100 diluted in PBS for 5 min and pre-incubated for 2 h in a blocking solution containing PBS, 0.1% sodium azide and 10% normal donkey serum (Sigma-Aldrich) at room temperature. Sections were then incubated with primary antibodies (Table 1) diluted in blocking solution for 48 h in a humidified chamber at 4 °C. Sections were washed in three changes of PBS for 15 min at room temperature and incubated with species-specific secondary antibodies (Table 1) diluted in PBS containing 0.1% sodium azide and 5% normal donkey serum for 4 h at room temperature. Sections were washed in three changes of PBS for 15 min at room temperature and mounted with Prolong Gold antifade mounting reagent (Invitrogen, Victoria, Australia).

To examine α_1-AR expression on nerve fibres, the tyramide signal amplification (TSA) method was used (25), allowing differentiation between primary antibodies raised in the same species. In these studies a biotin-conjugated TSA kit (NEL700; Perkin–Elmer Life Sciences, Victoria, Australia) was used to detect the pan-neuronal rabbit anti-PGP9.5 primary antibody, which was used at such a dilute concentration (1:400,000) that only TSA permitted subsequent detection. The second rabbit primary antibody against α_1-AR was used at normal concentration (1:250) and was detected by conventional immunohistochemistry. Endogenous peroxidase activity of tissue sections was quenched by incubation in 3% H_2O_2 in PBS for 10 min prior to application of blocking solution. Skin sections were incubated with the first primary antibody (anti-PGP9.5) overnight at room temperature in blocking solution. Sections were then consecutively incubated with biotin-conjugated donkey anti-rabbit IgG for 1 h (1:1000; Jackson ImmunoResearch, PA, USA), streptavidin-horse radish peroxidase in PBS for 30 min (1:150) followed by biotin-conjugated tyramide diluted 1:100 in amplification solution for 3 min. Visualisation was achieved using DyLight 549-conjugated streptavidin (diluted 1:1000 in PBS, 1 h; Jackson

ImmunoResearch, PA, USA). Between subsequent steps, sections were washed in three changes of PBS for 15 min.

Experimental controls

Controls for the specificity of the antibodies used in this study have been documented previously (20, 26, 27). To control for cross-reactivity between secondary antibodies, the procedures were performed as listed above except that one or other of the primary antibodies was omitted from each of the experimental protocols. Negative controls for the antisera included omission of the primary antibody or substitution of the primary antibody with donkey IgG serum. In addition, sections were treated with α_1-AR antibody (1:100) that had undergone pre-adsorption (12 h at 4 °C) with α_1-AR 339-349 blocking peptide (0.5 mg/ml, Abcam, Cambridge MA, USA). Pre-adsorption of the α_1-AR antiserum with α_1-AR specific blocking peptide resulted in elimination of immunostaining. Likewise, no immunostaining was observed when the α_1-AR antibody was replaced with donkey IgG serum.

For the TSA-amplified experiments, additional negative controls included omission of the α_1-AR antiserum from the second incubation, and PGP9.5 staining at a 1:400,000 dilution without the amplification process. For comparison between TSA-amplified and conventional immunostaining, one control section per run was immunostained with PGP9.5 antiserum diluted at 1:2000. Separate images from sections that had been double labelled with α_1-AR and PGP9.5 displayed identical results to the images of different sections that had been labelled separately for either α_1-AR or PGP9.5, except fewer fine individual nerve fibres could be visualised with the TSA method. Immunostaining was absent in negative controls of single antibody incubations or in negative controls of consecutively labelled sections following omission of primary antibodies. Incubation of control TSA-enhanced PGP9.5-immunolabelled sections with

anti-rabbit 488-conjugated fluorophore secondary antibodies failed to identify the PGP9.5-immunoreactive nerve fibres. Thus, it is unlikely that the PGP9.5 antibody at a concentration of 1:400,000 cross-reacted with the α_1-AR antibody.

Quantification of α_1-AR expression in skin

α_1-AR expression was quantified in the epidermis, sweat glands, blood vessels and nerve fibres by an investigator (ESD) who was blinded to side and group. Sweat glands and blood vessels were identified by SMA immunoreactivity, and nerve fibres were identified by PGP9.5 immunoreactivity. The epidermis was identified by morphology. Immunostained skin sections were imaged using a Leica TCS SP2 multiphoton confocal microscope. Two 200X magnification confocal image stacks were collected per section: one image of the papillary dermis and one image of the reticular dermis. Images were collected using identical laser power, gain and offset settings and each channel was imaged in a sequential manner to prevent overlap of emission spectra. Quantification was carried out using ImageJ image processing and analysis software (http://rsbweb.nih.gov/ij/), and was performed on the maximum projection of each confocal image stack. α_1-AR expression was measured as the average pixel intensity in a region of interest and was quantified in three sweat glands and all cross-sectional blood vessels in each image. α_1-AR staining intensity was also measured on nerve fibres in nerve bundles by creating a mask of PGP9.5 immunostaining in ImageJ, ensuring that consequent α_1-AR expression was examined only in those pixels that were also positive for PGP9.5 (Figure 1). Quantification of α_1-AR expression in the epidermis was performed by manually drawing around the epidermis and measuring the average α_1-AR staining intensity in the defined region.

<u>Statistical approach</u>

To permit data to be combined across multiple immunohistochemistry runs, α_1-AR$^+$ pixel intensity for each sample in each region of interest (the epidermis, sweat glands, blood vessels and dermal nerve fibres) was normalised within each run by expressing it as a z-score (i.e., with a mean of 0 and a standard deviation of 1). Preliminary analyses indicated that α_1-AR staining intensity was similar in the upper and lower limbs both on the affected and unaffected side of patients with CRPS; thus, data for all patients were pooled. Differences in α_1-AR staining intensity between patients and controls were investigated with the Mann-Whitney U test, and differences between CRPS-affected and contralateral limbs were examined with Wilcoxon's matched-pairs signed-rank test. Associations between α_1-AR immunoreactivity in the affected limb and CRPS duration, present pain intensity and symptom intensity were explored with Kendall's tau-b correlation coefficient. For these analyses, a pain rating of 1-3 was coded as "mild", 4-6 as "moderate" and 7-10 as "severe". In addition, CRPS for 6 months or less was coded as "acute", and longer than 6 months as "chronic".

Results

The majority of patients had suffered CRPS for extended periods (up to 230 months, mean 51 ± 57 months) but in six cases it had been present for six months or less. Pain intensity and symptoms associated with CRPS are listed in Table 2. Sympathetic blocks were administered once to eight patients and were administered two or more times to another six patients. In three of these patients, sympathetic blockade with onabotulinumtoxinA or radiofrequency lesions was followed by prolonged decreases in pain.

The localisation of α_1-ARs in the skin of humans was comparable to that described previously in animal tissues (20), with the receptors evident on blood vessels, sweat glands, keratinocytes and nerve fibres (Figures 1 and 2). α_1-AR staining was observed on nerve fibres in the papillary dermis in close proximity to the epidermal-dermal border, on fibres surrounding sweat glands in the reticular dermis and on fibres and the perineurium in nerve bundles (Figure 1).

α_1-AR staining was more intense in the epidermis both in the CRPS-affected (p = 0.009) and contralateral unaffected limb (p = 0.013) of patients than in the epidermis of controls (Figure 3). α_1-AR immunoreactivity was also more intense in nerve bundles in the reticular dermis both in the CRPS-affected (p = 0.039) and contralateral unaffected limb (p < 0.001) of patients than in controls (Figure 4). Differences between groups remained statistically significant after removing outliers (the single highest Z score in each group). Expression of α_1-ARs in blood vessels and the secretory coil of sweat glands was similar in patients and controls.

α_1-AR staining intensity in the epidermis of the CRPS-affected limb was associated with present pain intensity (Kendall's tau-b [N = 25] = 0.36, p = 0.025). A similar trend for α_1-AR staining intensity in nerve bundles in CRPS-affected skin did not achieve statistical significance (Kendall's tau-b [N = 15] = 0.33, p = 0.126). However, α_1-AR staining intensity in the symptomatic limb was not associated with CRPS duration or the graded intensity of sensory, motor or autonomic disturbances during the clinical examination (Table 3), and there was no clear difference in α_1-AR staining intensity between the three patients who obtained prolonged benefits from sympathetic blockade and the other patients. In addition, there was no association between CRPS symptoms and α_1-AR staining intensity in the contralateral limb.

Discussion

We reported recently that α_1-ARs were expressed more strongly on nociceptive fibres in the affected than contralateral hind paw of rats after partial sciatic nerve ligation and chronic constriction injury (21, 22), and were expressed more strongly on nerve fibres and keratinocytes in the affected than contralateral unaffected limb of patients with CRPS type II (22). Thus, of particular note in the present study was the heightened expression of α_1-ARs on dermal nerve fibres and epidermal cells both in the affected and contralateral unaffected limb of patients with CRPS type I. These findings may reflect a systemic response to injury or a pre-existing vulnerability to CRPS. If so, this particularly implicates dermal nerve fibres and epidermal cells in the pathophysiology of CRPS, as α_1-AR staining intensity on cells in sweat glands and cutaneous blood vessels was similar in patients and controls. This significantly extends our previous radioligand-binding experiments, where we were able to demonstrate an augmented expression of cutaneous α_1-ARs in the hyperalgesic skin of CRPS patients compared with controls but were unable to localise the α_1-ARs to specific skin strata or, in particular, to nerve fibres (19).

After peripheral nerve injury, an increased expression of α_1-ARs on nociceptive afferents has been shown to contribute to neuropathic pain behavior in animals (28-30), but whether this mechanism also plays a role in CRPS type I is unclear. In the present study, expression of α_1-ARs was increased on cutaneous nerves not only in the CRPS-affected limb but also contralaterally. Sensory disruption, neurovascular disturbances and inflammatory changes in CRPS type I frequently involve both the affected and contralateral limb (10, 31-37), possibly due to systemic inflammatory responses, "mirror image" neuropathic changes (38-41) or an underlying predisposition. For example, in a study by Mailis-Gagnon and Bennett (10), injection of the α_1-AR agonist phenylephrine evoked abnormal pain in the limb contralateral to the symptomatic limb of three patients

with CRPS. Importantly, substance P-induced protein extravasation and neurogenic inflammation are increased bilaterally in CRPS (31, 32, 36). Hence, a cascade of inflammatory responses initiated by injury may induce neurovascular disturbances in the CRPS-affected limb which, in turn, exacerbate pathophysiological responses to injury (42-44). Conceivably, heightened expression of α_1-ARs on dermal nerve fibres could contribute to this process in the injured limb as their stimulation generates axon reflexes both in animal preparations and in human skin (45-49).

The heightened expression of α_1-ARs in the epidermis of CRPS type I patients might also augment inflammatory disturbances after limb injury. This up-regulation is limited to the injured limb in patients with CRPS type II (22) whereas, in CRPS type I, it appears to include epidermal cells both the affected and unaffected contralateral limb. Whether this represents an underlying predisposition or a systemic response to injury is not yet established but, in either case, has clear pathophysiological implications. The epidermis forms part of an important barrier to the external environment that is involved in sensory transduction and inflammatory reactions to injury. Keratinocytes make up about 90% of the cells in the epidermis and can influence nociception by releasing ligands that act on epidermal sensory nerve fibres. For instance, an elevation of sodium channel expression in the keratinocytes of patients with CRPS and post herpetic neuralgia (50) may lead to increased epidermal ATP release and excessive activation of P2X purinergic receptors on primary nociceptive afferents. In addition, the neuropeptide calcitonin gene-related peptide (CGRP) β is elevated in keratinocytes in various animal models of neuropathic pain and may also be elevated in patients with CRPS (51). Engrafting human keratinocytes into an injured rat sciatic nerve increases the excitability of regenerating axonal sprouts in association with heightened production of nerve growth factor from the transplanted keratinocytes, resulting in chronic pain behaviors (52). Similarly, in the

distal tibia fracture model of CRPS, fracture triggers the expression and release of pro-inflammatory cytokines and nerve growth factor from keratinocytes (53), and blocking the actions of these cytokines reduces signs of pain (54-57). In lymphocytes, expression of the α_{1A}-AR subtype is driven by inflammatory mediators (58); conversely, exposure to noradrenaline increases the production of pro-inflammatory mediators in cells that express α_1-AR (59-61). Our findings raise the prospect of similar effects in keratinocytes, as the intensity of α_1-AR staining in the CRPS-affected epidermis was proportional to pain intensity.

Apart from this association, α_1-AR expression in the CRPS-affected tissue was unrelated to the graded intensity of clinical symptoms during the clinical examination. However, we may have overlooked some associations because sensory and autonomic disturbances were not examined quantitatively in laboratory investigations. This applies particularly to hyperhidrosis and vasomotor disturbances, which are influenced strongly by ambient temperature and emotional states. Thus, functional studies involving activation of α_1-ARs are required to further clarify their role in CRPS type I.

The distribution of α_1-ARs on dermal blood vessels was similar in patients and controls. Spontaneous pain and mechanical hyperalgesia intensify during whole body cooling in CRPS patients with "sympathetically maintained pain" (62), possibly due to microvascular pathology that produces ischaemia and inflammation (23, 63, 64). In patients with CRPS, constriction of superficial hand veins to infusion of noradrenaline is greater than normal, consistent with heightened vascular expression of α-adrenoceptors (65). However, in the present study, the distribution of α_1-ARs on dermal blood vessels was similar in patients and controls. It would be of interest in future studies to assess whether the distribution of α_1-ARs on dermal blood vessels and other cellular targets is altered in patients with signs of sympathetically maintained pain (i.e., those who benefit

from sympathetic blockade or whose pain responds to intradermal injection of adrenergic agents). Unfortunately, this could not be examined in the present study, as only a few patients clearly benefited from sympathetic blockade.

In the present study, α_1-AR staining intensity was similar in the sweat glands of patients and controls. It has been shown that axon-reflex sweating to iontophoresis of the α_1-AR agonist phenylephrine was greater in the affected limb of patients with acute CRPS type I compared to controls and patients who had recovered from CRPS (66). While this suggests that a heightened α_1-AR expression on sudomotor nerve fibres or the sweat glands themselves might drive hyperhidrosis, we were unable to explore this as sweating was not investigated quantitatively in our study.

Some additional limitations apply to this study. For example, one-quarter of the patients rated pain as mild during the two weeks prior to the skin biopsy, and the majority had pain for longer than six months. As sympathetically maintained pain appears to be more common in the acute than chronic stages of CRPS (11), α_1-AR staining intensity might also be greater in patients with acute than chronic pain. In most of our patients, symptoms were confined to the injured limb. However, we may have overlooked subclinical contralateral disturbances associated with α_1-AR up-regulation. Quantitative sensory and physiological studies involving comparisons with healthy controls will be required to resolve this. Finally, α_1-AR staining was investigated in the epidermis as a whole, based on morphology. Although most cells in the epidermis are keratinocytes, in future studies it would be important to identify these cells with specific markers of keratin.

In conclusion, α_1-ARs were up-regulated bilaterally in the dermal nerves and keratinocytes of patients with CRPS type I. This heightened α_1-AR expression might increase vulnerability to CRPS type I and/or contribute to neuroinflammatory

disturbances and pain by augmenting the excitability of these cells. If so, topical treatments that target up-regulated α_1-ARs could be beneficial in relieving allodynia and pain in patients with CRPS type I.

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Table 1. Primary and secondary antibodies: dilutions and source

Antigen and Host Species	Dilution	Product Code	Source
Primary Antibodies			
Anti α_1-AR: Rabbit polyclonal	1:250	A270	Sigma-Aldrich, MO. USA
Anti PGP9.5: Rabbit polyclonal	1:400000	7863-0504	AbD Serotec, NC. USA
Anti SMA: Mouse monoclonal	1:800	A2547	Sigma-Aldrich, MO. USA
Secondary Antibodies			
DyLight-488: Donkey anti-rabbit IgG	1:600	711-485-152	Jackson ImmunoResearch, PA. USA
DyLight-549: Donkey anti-rabbit IgG	1:1200	711-506-152	Jackson ImmunoResearch, PA. USA
DyLight-488: Donkey anti-mouse IgG	1:600	715-486-151	Jackson ImmunoResearch, PA. USA
Cy3: Donkey anti-mouse IgG	1:750	715-166-152	Jackson ImmunoResearch, PA. USA

Abbreviations: α_1-Adrenergic Receptor (α_1-AR), Protein gene product-9.5 (PGP9.5), Smooth Muscle Actin (SMA), Indocarbocyanine (Cy3).

Table 2. Clinical characteristics in patients with CRPS type I[a]

	CRPS type I (N = 31)
Pain intensity (0-10)	5.6 ± 2.7
mild	26%
moderate	29%
severe	45%
Duration (months ± S.D.)	51 ± 57
up to 6 months	19%
longer than 6 months	81%
Trophic changes	26%
Blunting to scratching	
none	34%
mild	38%
severe	28%
Motor disturbances	
mild	47%
severe	53%
Allodynia to tapping	
none	3%
mild	36%
severe	61%
Swelling	
none	38%
mild	55%
severe	7%
Sweating	
symmetrical	72%
increased in the affected limb	28%
Temperature	
affected limb cool	68%
symmetrical	6%
affected limb warm	26%
Colour changes	
affected limb cyanotic	48%
symmetrical	27%
affected limb flushed	34%

[a]Symptoms were also noted in the contralateral limb of three patients (10% of the sample). In one patient this involved tightness in the contralateral knee, hypoaesthesia in the contralateral thigh and minor swelling of the contralateral foot. In another, both feet were flushed and sensitive to mechanical stimulation but pain was greater and extended throughout the entire limb on the injured side. In the third patient, pain and allodynia were present in all four limbs, but pain and other symptoms were much more severe in the injured limb than elsewhere.

Table 3. Association (Kendall's tau-b) between severity of CRPS symptoms (mild, moderate or severe) and α_1-AR staining intensity

	α_1-AR staining intensity in the symptomatic limb			
	Epidermis **(N=25)**	**sweat glands** **(N=14)**	**blood vessels** **(N=20)**	**Nerves** **(N=15)**
Duration greater than 6 months	.13	-.04	-.09	-.12
Pain	.36*	-.24	-.12	.33
Allodynia	.22	.11	.03	-.09
Sensory blunting to scratching	.22	.17	-.19	.19
Swelling	.18	-.04	.18	.37
Motor disturbances	.14	-.14	-.18	.16

* $p < 0.05$; ** $p < 0.01$

Figure legends

Figure 1 α_1-AR immunoreactivity on nerve fibres in human skin. A-D and E-H show α_1-AR expression in cross- and longitudinal sections of nerves in the lower dermis. I-L show α_1-AR expression on a nerve fibre in the upper dermis and M-P show α_1-AR expression on nerve fibres encircling a sweat gland (SG). α_1-AR is shown in green and PGP9.5 is shown in red. C, G, K and O show merged images of α_1-AR and PGP9.5 staining, and D, H, L and P show in white all pixels that positively express α_1-AR and PGP9.5 staining defined by the ImageJ plugin-in "Colocalisation Finder".

Figure 2 α_1-AR immunoreactivity was observed in keratinocytes (A), blood vessels (B-D) and sweat glands (E-G) in human skin. α_1-AR is shown in green, smooth muscle actin is shown in red and D and G show merged images of α_1-ARs and smooth muscle actin on blood vessels and sweat glands respectively.

Figure 3 α_1-AR immunoreactivity in the epidermis of human skin. Top panels illustrate α_1-AR staining in epidermis of skin biopsy samples from a control and the affected and unaffected limbs of a patient with CRPS. The bottom panel illustrates data from all samples expressed as z-scores. When averaged across all patients, there was a significant difference between controls and CRPS samples (# $p < 0.05$ between healthy controls and both affected and contralateral sides in patients with CRPS type I).

Figure 4 Differences between groups in α_1-AR expression on nerve fibres in nerve bundles in the reticular dermis. (A-I) Representative images of nerve bundles identified by staining to PGP9.5 (red) showing that α_1-AR expression (green) was enhanced both on

the affected and contralateral unaffected side in patients with CRPS compared with controls. The bottom panel illustrates data from all samples expressed as z-scores. Analysis of these scores with the Mann-Whitney U test indicated that α_1-AR expression was significantly greater in nerve bundles both in the affected and the contralateral unaffected reticular dermis of CRPS patients than in controls (# $p<0.05$). α_1-AR expression was tabulated only in those pixels that were also positive for PGP9.5.

| | α₁-AR | PGP9.5 | Merge | Co-localisation |