Peripheral cholinergic function in humans with chronic fatigue syndrome, Gulf War syndrome and with illness following organophosphate exposure

Faisel KHAN, Gwen KENNEDY, Vance A. SPENCE, David J. NEWTON and Jill J. F. BELCH

Vascular Diseases Research Unit, University Department of Medicine, Ninewells Hospital and Medical School, Dundee DD1 9SY, Scotland, U.K.

A B S T R A C T

In the present study, we have investigated whether the peripheral cholinergic abnormalities that we have reported previously [Spence, Khan and Belch (2000) Am. J. Med. **108**, 736–739] in patients with chronic fatigue syndrome (CFS) are also present in those with Gulf War syndrome (GWS) and agricultural workers exposed to organophosphate pesticides, where cholinesterase inhibition is specifically implicated. We also looked at whether these abnormalities might be due to a reduction in the activity of cholinesterase expressed on the vascular endothelium. We used laser Doppler imaging to measure the forearm skin blood flow responses to iontophoresis of acetylcholine and of methacholine (which is resistant to breakdown by cholinesterase) in patients with CFS, GWS and those with a history of ill health after definite organophosphate exposure, as well as in matched healthy controls. The response to acetylcholine was significantly higher in patients with CFS than in controls ($P = 0.029$, repeated-measures ANOVA), but was normal in those with GWS and those exposed to organophosphates. The methacholine response was higher than the acetylcholine response in all patient groups except for those with CFS, where there was no difference between the responses. Although there are many clinical similarities between these three illnesses, our results indicate peripheral cholinergic abnormalities in the vascular endothelium of only patients with CFS, suggesting that this syndrome has a different aetiology, which might involve inhibition of vascular cholinesterase.

INTRODUCTION

There are many clinical similarities between the symptom complex known as chronic fatigue syndrome (CFS) and the symptoms reported by those with Gulf War syndrome (GWS). Indeed, it has been suggested that many patients with GWS fulfil specific criteria for CFS [1–3], often with less severe symptoms [4]. Fatigue, muscular pain, cognitive problems, changes in intestinal function, disturbed sleep, temperature dysregulation and disorders of the autonomic nervous system are common in both syndromes, and closely resemble conditions associated with impaired cholinergic mechanisms [5]. GWS has been attributed to exposure to cholinesterase inhibitors, such as pyridostgmine bromide (used as nerve agent protection) and insecticides, employed during the 1991 Gulf War conflict [6]. A similar illness has also been described in agricultural workers exposed to the cholinesterase inhibitors contained in organophosphate insecticides [7].

Key words: cholinergic function, chronic fatigue syndrome, Gulf War syndrome, microcirculation, laser Doppler imaging, organophosphate.

Abbreviations: CFS, chronic fatigue syndrome; GWS, Gulf War syndrome; OPE, organophosphate exposure.

Correspondence: Dr Faisel Khan (e-mail f.khan@dundee.ac.uk).

Most research investigating cholinergic abnormalities has focused on the central nervous system of patients with GWS [8] and those exposed to organophosphates [9]. However, in a previous report [10], we described evidence of peripheral cholinergic dysfunction in patients with CFS. Skin microvascular responses to acetylcholine were higher than normal and, as nitric oxide-mediated responses were normal, we hypothesized that this vascular hypersensitivity was specific to a cholinergic pathway, and perhaps due to a reduction in vascular cholinesterase activity. This would prolong the binding of acetylcholine to muscarinic receptors on the vascular endothelium. Support for this hypothesis comes from the finding that direct cutaneous application of organophosphate chemicals, such as malathion, also causes vascular hypersensitivity to acetylcholine [11]. Furthermore, when the cholinesterase inhibitor edrophonium is applied to forearm skin, the decay of acetylcholine-stimulated hyperaemia is significantly prolonged [12], providing a link between vascular cholinesterase activity and the dynamics of acetylcholine-induced blood flow responses.

The primary aim of our present study was to determine whether the abnormal vascular response to acetylcholine reported previously by us in CFS [10] is also present in patients with GWS and those exposed to organophosphates, given that cholinesterase inhibition is specifically implicated in their chronic ill health.

Our second aim was to test further the hypothesis that vascular hypersensitivity in CFS is due to a reduction in cholinesterase activity. We investigated this by measuring the microvascular responses to methacholine. This vasodilator is almost identical with acetylcholine, but is much less influenced by the action of cholinesterase. Therefore, if our previous results were related to an abnormality of vascular cholinesterase activity, we would expect to find no difference between the vascular responses to methacholine and those to acetylcholine.

METHODS

Participants

We recruited 141 volunteers for this study, which was approved by the Tayside Committee on Medical Research Ethics, and conducted in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Each volunteer gave written informed consent to take part.

The participants were divided into four groups. The CFS group consisted of 52 patients diagnosed with CFS (myalgic encephalomyelitis). They were selected randomly from a cohort of previously studied patients.

The GWS group consisted of 24 patients, and these were veterans of the Gulf War who had developed chronic-fatigue-like symptoms. All patients had reported taking the cholinesterase inhibitor pyridostigmine bromide as nerve agent protection and reported possible exposure to one of a number of organophosphate insecticides used routinely at the time of the conflict in early 1991. They were recruited from a register of patients held by the Gulf War Veterans and Families Association, and all had symptoms classified as moderate to severe.

The organophosphate exposure (OPE) group contained 25 patients with chronic-fatigue-like symptoms who had self-reported a definite history of contact with organophosphate pesticides as the cause of their illness. Of these patients, 24 had been exposed to organophosphate-containing sheep dip, and the other patient reported occupational exposure to organophosphates in the fish farm industry. They were recruited from registers of patients held by the Organophosphate Information Network Scotland and the Pesticide Action Network U.K.

In addition, we recruited 40 healthy volunteers to form a pool from which to select control groups for each of the patient groups, matched for age, sex and smoking habits.

All participants underwent a thorough medical examination. Six patients in the CFS group were excluded: one had diabetes, one had a possible neurological condition, one had angina, one was unable to comply with the tests, and two did not fulfil the criteria for CFS [13]. One patient in the OPE group was excluded because of lymphoma. All 94 remaining patients fulfilled the Fukuda 1994 Centres for Disease Control classification for CFS [13].

The demographics for all participants are recorded in Table 1, along with the prevalence of specific symptoms in each of the three patient groups.

Protocol

The experiments were conducted in a laboratory at an environmental temperature of $22 \pm 1\,^{\circ}C$, and the participants were seated with their arms supported at heart level. We started by measuring systolic and diastolic blood pressure, and taking a venous blood sample from which to measure levels of cholesterol, in order to exclude hypercholesterolaemia as a cause of vascular damage.

We assessed peripheral cholinergic function by measuring skin blood flow responses to iontophoresis of acetylcholine and methacholine. Iontophoresis is a drug-delivery method which stimulates the migration of charged ions across the skin in a non-invasive manner and without inducing systemic effects.

We prepared measurement sites on the surface of the forearm by removing surface keratinocytes with adhesive tape and cleaning the area with an alcohol swab. The iontophoresis chamber (Moor Instruments Ltd, Axminster, Devon, U.K.), which is a Perspex ring of diameter 20 mm with a wire ring running round its inner surface, was fixed to the skin with adhesive tape. The hydrochloride salt of each drug (Sigma–Aldrich,

Table 1 Demographics, prevalence of symptoms of CFS and clinical measures in CFS, GWS and OPE patients and in matched controls

Blood pressure (BP) and cholesterol results are presented as means ± S.D. CON_{CFS}, CON_{GWS} and CON_{OPE} are matched controls for the CFS, GWS and OPE patients respectively.

Parameter	CFS	CON_{CFS}	GWS	CON_{GWS}	OPE	CON_{OPE}
n	46	31	24	17	24	17
Mean age (years) (range)	48 (19–63)	44 (19–49)	39 (31–50)	40 (29–53)	48 (33–64)	46 (30–59)
Men/women	17/29	11/20	23/1	16/1	21/3	14/3
Smokers	6 (+9 ex)	5 (+2 ex)	12 (+4 ex)	8	2 (+4 ex)	3 (+2 ex)
Symptoms						
Chronic fatigue	100%	–	100%	–	100%	–
Sore throat	52%	–	48%	–	46%	–
Cognitive symptoms	100%	–	96%	–	100%	–
Tender lymph nodes	56%	–	24%	–	38%	–
Muscle pain	94%	–	80%	–	96%	–
Multi-joint pain	77%	–	76%	–	96%	–
Headaches	58%	–	68%	–	88%	–
Unrefreshing sleep	90%	–	72%	–	96%	–
Post-exertional malaise	100%	–	96%	–	96%	–
Systolic BP (mmHg)	125 ± 21	123 ± 17	124 ± 9	125 ± 11	135 ± 21	125 ± 11
Diastolic BP (mmHg)	74 ± 12	72 ± 10	80 ± 10	76 ± 12	78 ± 11	76 ± 10
Cholesterol (mmol/l)	5.18 ± 1.03	5.18 ± 0.95	5.42 ± 1.03	5.08 ± 0.89	5.42 ± 0.85	5.13 ± 0.78

Poole, Dorset, U.K.) was dissolved in deionized water to a concentration of 10 g/l, and approx. 2 ml of this solution was used to fill the chamber. The positive lead of a current source was connected to the electrode, and the negative lead was attached to a conductive hydro-gel pad on the wrist, which served as the reference electrode.

The drugs were delivered consecutively at different sites on the arm. We used a delivery current of 100 μA and administered each drug as a successive accumulation of 10, 20, 40 and 80 s doses; effectively 1, 2, 4 and 8 mCoulombs (mC).

Cutaneous perfusion was assessed at each delivery site using laser Doppler imaging (moorLDI; Moor Instruments). Briefly, a 2 mW helium–neon laser scans the surface of the skin and light which is backscattered from moving erythrocytes undergoes a shift in frequency proportional to their velocity, according to the Doppler principle [14]. The resulting colour-coded image represents skin blood flow over the scan area; a relative measure called the laser Doppler flux. As the instrument has a catchment depth of approx. 1 mm [15], this measurement derives mainly from the microcirculation of the papillary dermis which, on average, contains vessels varying in diameter from 10–50 μm [16].

After each iontophoretic delivery, the solution was removed and the chamber dried, and then images were recorded at 30 s intervals. We recorded three scans after each acetylcholine dose and four scans after each methacholine dose (as this response lasted longer). The laser head was positioned 50 cm from the skin surface and the scan region, encompassing the iontophoresis chamber, was approx. 8 cm × 8 cm. We used a spatial resolution of approx. 1 mm/pixel and a scan speed of 4 ms/pixel.

This combination of iontophoresis and laser Doppler imaging has been used successfully by us [17] and others [18] in many studies, and we [19] recently confirmed the good reproducibility of the technique for acetylcholine and methacholine.

Analysis

The images were analysed using dedicated software, and we calculated the median laser Doppler flux over the delivery site for each. For each dose response, the mean of the two highest flux values was taken and was divided by the baseline measurement to give a ratio representing the change in flow.

The Shapiro–Wilks test indicated that our blood flow measurements did not follow a normal distribution. The distribution of the $\log_{10}$-transformed data was not significantly different from normal, however, and we therefore used repeated-measures ANOVA (SPSS Inc, Chicago, IL, U.S.A.) on these data to determine the statistical significance of differences between the patient groups and between the acetylcholine and methacholine responses. We used post-hoc significance testing (Student's *t* tests) to determine at which doses any differences existed. The blood pressure and cholesterol data were analysed using one-way ANOVA. Statistical significance was acknowledged if the probability of a type-1 error was less than 5% (i.e. $P < 0.05$).

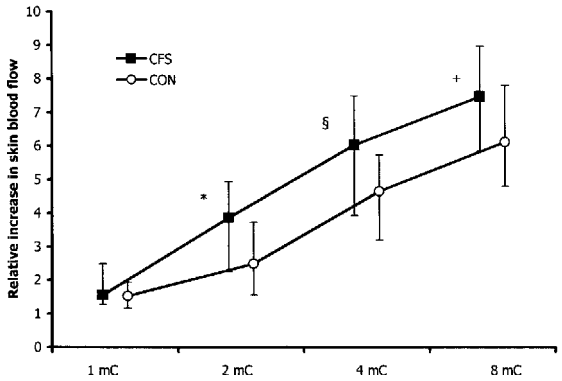

Figure 1 Forearm skin blood flow responses to iontophoresis of four cumulative doses of acetylcholine in patients with CFS and in matched controls

Results are expressed as medians with 25th and 75th percentiles. $^{*}P = 0.016$, $\S P = 0.024$ and $^{+}P = 0.044$ compared with controls. CON, control.

Table 2 Forearm skin blood flow responses to iontophoresis of four cumulative doses of acetylcholine in patients with GWS and OPE and in matched controls

Data are median (inter-quartile range) changes in blood flow relative to baseline. CON_{GWS} and CON_{OPE} are matched controls for the GWS and OPE patients respectively.

	Relative changes in blood flow			
Dose (mC)	GWS	CON_{GWS}	OPE	CON_{OPE}
1	1.28 (0.48)	1.20 (0.53)	1.55 (1.29)	1.28 (0.58)
2	2.43 (1.15)	1.89 (1.85)	3.05 (2.81)	2.36 (2.02)
4	3.56 (1.77)	3.67 (2.67)	4.13 (4.33)	4.64 (2.17)
8	4.92 (1.66)	5.28 (2.11)	5.50 (3.79)	5.84 (2.12)

RESULTS

Table 1 shows the prevalence of physical symptoms in each patient group. There was no significant difference between the subject groups in either systolic blood pressure ($P = 0.121$), diastolic blood pressure ($P = 0.082$) or cholesterol ($P = 0.744$).

Iontophoresis of both acetylcholine and methacholine produced dose-dependent increases in skin blood flow in all participants studied. Figure 1 shows that the response to acetylcholine was significantly higher in the CFS patients than in their control group ($P = 0.029$), and this was by 22 % at the highest dose. There were no such differences between controls and GWS ($P = 0.540$) or OPE ($P = 0.879$; Table 2) patients.

There were no significant differences from control in the methacholine response for the CFS ($P = 0.132$), GWS ($P = 0.484$) or OPE ($P = 0.069$) groups.

Methacholine produced a higher skin blood flow response than did acetylcholine in the pooled controls

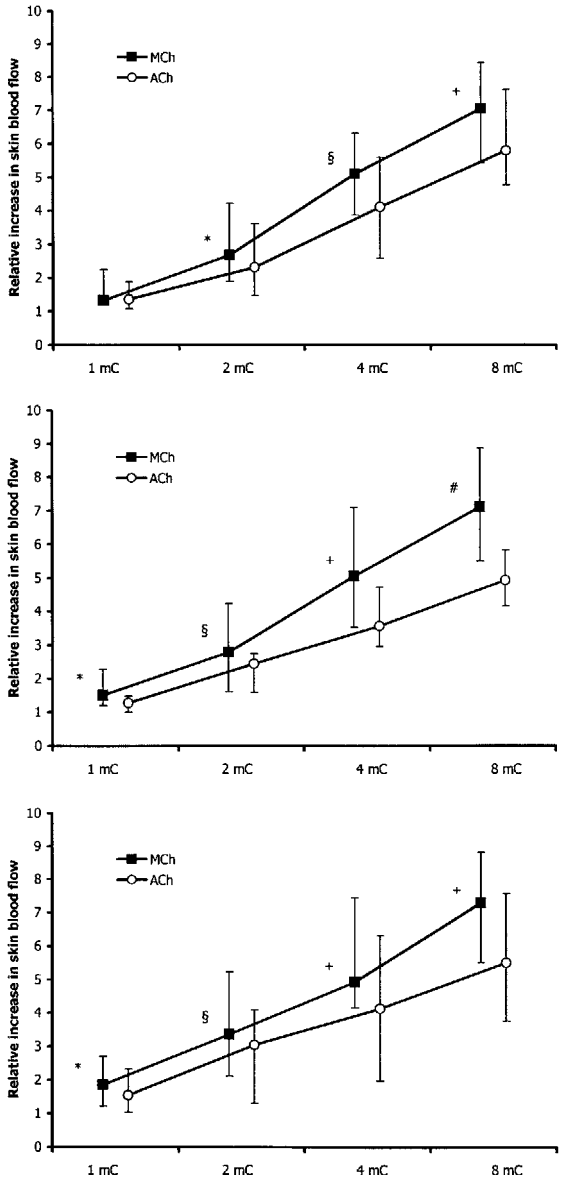

Figure 2 Forearm skin blood flow responses to iontophoresis of four cumulative doses of methacholine (MCh) and acetylcholine (ACh) in healthy controls (top) and patients with GWS (middle) and OPE (bottom)

Results are expressed as medians with 25th and 75th percentiles. In the top panel, $^{*}P = 0.033$, $\S P = 0.024$ and $^{+}P = 0.006$ when methacholine is compared with acetylcholine; in the middle panel, $^{*}P = 0.007$, $\S P = 0.042$, $^{+}P = 0.012$ and, $\#P < 0.001$ when methacholine is compared with acetylcholine; and in the bottom panel, $^{*}P = 0.034$, $\S P = 0.010$, $^{+}P < 0.001$ when methacholine is compared with acetylcholine.

($P = 0.022$; Figure 2, top panel) and GWS ($P = 0.001$; Figure 2, middle panel) and OPE ($P < 0.001$; Figure 2, bottom panel) groups. In the patients with CFS, however, the difference between acetylcholine and methacholine was not statistically significant (median dose responses to acetylcholine of 1.55, 3.86, 6.02 and 7.45, compared

with responses to methacholine of 1.63, 3.83, 6.63 and 8.23; $P = 0.488$).

DISCUSSION

In the present study, we have confirmed in a larger group our previous finding [10] of enhanced sensitivity to acetylcholine in the peripheral cutaneous circulation of patients with CFS. We have also shown that the skin blood flow response to methacholine in these patients is not significantly different from the response to acetylcholine. Methacholine is almost identical with acetylcholine, except that it is affected much less by the action of cholinesterase, an enzyme which limits the effects of acetylcholine by removing it from its muscarinic receptors. The actions of methacholine are therefore prolonged compared with those of acetylcholine. Although endothelial dysfunction would influence the responses to both acetylcholine and methacholine, an abnormality in cholinesterase activity should predominantly affect the response to acetylcholine, with minimal effect on the methacholine response. Since we found no difference between acetylcholine and methacholine responses in the CFS group, the results support our hypothesis that there is an abnormality of cholinergic activity, perhaps involving cholinesterase, in the vascular bed of patients with CFS.

There are a number of potential mechanisms by which iontophoretic application of acetylcholine might be affecting skin blood flow, raising the possibility that these effects are not solely related to the endothelium. One of the possible effects of acetylcholine is to inhibit neural release of noradrenaline. However, neural blockade has been shown to have no significant effect on the forearm skin response to acetylcholine [20,21], indicating that neural influences do not play a major role in acetylcholine-mediated vasodilatation. The contribution of sympathetic nerves to blood flow changes in forearm skin is relatively small. Pergola et al. [22] showed that release of adrenergic vasoconstrictor tone contributes only minimally to the overall rise in skin blood flow following direct heating. Sympathetic innervation is denser only in acral regions, such as the hands and feet, and can have a greater effect on skin blood flow here. It is also unlikely that acetylcholine is causing vasodilatation through stimulation of sweat glands and subsequent release of vasoactive kinins. We never see forearm sweating following this protocol. We therefore conclude that the predominant mechanism of cholinergic vasodilatation observed in our present study is mediated through the endothelium.

Our present results do not support the existence of endothelial cholinergic abnormalities in patients with GWS or those exposed to the effects of organophosphates. The blood flow responses to acetylcholine and methacholine were not significantly different from control in either group, and acetylcholine responses were below those for patients with CFS.

It should be stressed that sensitivity to acetylcholine is not diagnostic of CFS, just as insensitivity to acetylcholine in illnesses such as diabetes and vascular disorders is not diagnostic of those conditions. However, our present data show that the trend in patients with CFS is to have a blood vessel response to acetylcholine that is greater than that of matched control subjects and, in that fact alone, this finding is unique to the illness.

These present results raise interesting questions about the aetiology of these different syndromes. All three have a similar clinical picture, and patients with CFS and GWS are equally disabled on accepted standardized measures of physical functioning. Other indicators which might influence endothelial function, such as measures of blood pressure and serum cholesterol, are the same in all three groups and are no different from controls.

There is already evidence of differences in the cholinergic pathway between patients with CFS and GWS. Magnetic resonance spectroscopy in patients with CFS has revealed a high choline/creatinine ratio, indicating increased levels of free choline, in areas of the central nervous system [23,24]. In GWS, however, the choline/creatinine ratio in brain structures is normal or depressed [25]. An increased choline peak on magnetic resonance spectroscopy is a reflection of abnormal membrane phospholipid metabolism and is normally associated with an infectious [26,27] or inflammatory state [28]. On the other hand, a reduced choline/creatinine ratio, as seen in GWS, indicates atrophy [29,30], late infarction [31] or neuronal damage to the brainstem [25]. These data reflect our present findings in the peripheral vascular endothelium, where the response to acetylcholine was increased in CFS and normal in GWS and in patients exposed to organophosphates.

In a recent study [32], we found that, following cutaneous stimulation with acetylcholine, the blood flow response took longer to return from peak to baseline in patients with CFS. This prolongation of the acetylcholine response could be explained by under-expression of cholinesterase on endothelial cells [33]. We speculated that this might be a consequence of a viral infection, since cholinesterase is inhibited within cholinergically sensitive cells when infected with herpes simplex virus-type 1 [34] and, in the case of lymphocytic choriomeningitis virus, such inhibition in neuroblastoma cells persists for years after infection [35].

CFS shares many symptoms with several other conditions [36,37]; however, although patients with CFS, GWS, and OPE all exhibit symptoms which are indistinguishable within the framework of the Fukuda 1994 Centres for Disease Control criteria for CFS, we have found significant differences in their blood vessel responses to acetylcholine. Hypersensitivity

was seen only in the patients with CFS (myalgic encephalomyelitis), and this may be related to a reduction in acetylcholinesterase expressed on the vascular endothelium. The evidence in the present study points to the fact that CFS involves a different vascular abnormality to GWS and organophosphate-induced chronic illness, especially in terms of abnormalities of the choline pathways and their potential effects on vascular integrity. This suggests that these illnesses also have different aetiologies.

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