European Journal of Pain xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

European Journal of Pain

journal homepage: www.EuropeanJournalPain.com

Mitochondrial dysfunction in muscle tissue of complex regional pain syndrome type I patients

Edward C.T. Tan [a,1], Antoon J.M. Janssen [b,1], Peggy Roestenberg [c], Lambert P. van den Heuvel [b], R. Jan A. Goris [a], Richard J.T. Rodenburg [b,*]

[a] Departments of Surgery, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands
[b] Departments of Pediatrics, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands
[c] Departments of Biochemistry, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

ARTICLE INFO

Article history:
Received 1 November 2010
Accepted 18 December 2010
Available online xxxx

Keywords:
CRPS I
Reactive oxygen species
Mitochondria
Energy metabolism
Skeletal muscle

ABSTRACT

Reactive oxygen species (ROS) are known to be involved in the pathophysiology of complex regional pain syndrome type I (CRPS I). Since the mitochondrial respiratory chain is a major source of ROS, we hypothesized that mitochondria play a role in the pathophysiology of CRPS I. The hypothesis was tested by studying mitochondrial energy metabolism in muscle tissue from amputated limbs of CRPS I patients. We observed that mitochondria obtained from CRPS I muscle tissue displayed reduced mitochondrial ATP production and substrate oxidation rates in comparison to control muscle tissue. Moreover, we observed reactive oxygen species evoked damage to mitochondrial proteins and reduced MnSOD levels. It remains to be established if the mitochondrial dysfunction that is apparent at the end-stage of CRPS I is also present in earlier stages of the disease, or are secondary to CRPS I. The observation of a reduced mitochondrial energy production combined with reactive oxygen species induced damage in muscle tissue from CRPS I patients warrants further studies into the involvement of mitochondrial dysfunctioning in the pathophysiology of CRPS I.

1. Introduction

Complex Regional Pain Syndrome Type I (CRPS I) is a severe, disabling, and painful disease which may occur in an extremity after a trauma or injury. In the Dutch population the estimated incidence is 4000 per year, which includes approximately three times more females than males (De Mos et al., 2007). Clinical features include spontaneous and stimulus-evoked pain, edema, vasomotor and sudomotor disturbances, motor dysfunction, and trophic changes, without evidence for peripheral nerve injury according to IASP criteria (Mersky and Bogduk, 1994; Stanton-Hicks et al., 1995. Despite growing knowledge of the pathophysiology of CRPS I, the primary cause of the syndrome is still unknown. In a recent study of 41 randomized controlled trials on treatment of CRPS I only limited evidence to formulate recommendations for treatment was found (Tran de et al., 2010). Possibilities for therapeutic intervention are therefore limited and include pharmacological pain relief, anti-inflammatory treatment, treatment with biphosphonates (Robinson et al., 2004) or with free radical scavengers (Perez et al., 2003; van der Laan et al., 1998; van der Laan and Goris, 1997), and physical therapy. There are indications for an impaired oxygen metabolism and mitochondrial dysfunction in skeletal muscle of CRPS I patients (Goris, 1998; Heerschap et al., 1993). In physiological processes like maturation, and pathological circumstances like tumorigenesis, cells are exposed to hypoxia or reduced oxygen levels. Under these circumstances elevated levels of reactive oxygen species (ROS) are produced, acting as second messengers by activating hypoxia-inducible factors (HIFs) that stimulate the cells to maintain normal ATP synthesis (Klimova and Chandel, 2008). Under hypoxic conditions, muscle tissue fails to maintain a normal redox state, resulting in an increased production of ROS, and subsequent cell injury or dysfunction (Clanton, 2007). Serum lipid peroxidation products and salivary antioxidative parameters, known to be induced by ROS, are increased in CRPS I patients (Eisenberg et al., 2008). Increased ROS production can be a consequence of dysfunction of the mitochondrial respiratory chain, an essential part of the mitochondrial energy generating system (MEGS). Although muscle tissue analysis is regarded as the golden standard to study the MEGS (Rodenburg,

* Corresponding author. Address: Radboud University Nijmegen Medical Center, Nijmegen Center for Mitochondrial Disorders, Department of Pediatrics, Laboratory of Genetic, Endocrine and Metabolic Disease, PO Box 9101, 6500GA Nijmegen, The Netherlands. Tel.: +31 24 361 4818; fax: +31 24 361 8900.
E-mail address: r.rodenburg@cukz.umcn.nl (R.J.T. Rodenburg).
URL: http://www.ncmd.nl (R.J.T. Rodenburg).
[1] Contributed equally to this study.

1090-3801/$36.00 © 2010 European Federation of International Association for the Study of Pain Chapters. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejpain.2010.12.003

2010), it is not advisable to perform a muscle biopsy on CRPS I patients because of the risk of either recurrence of CRPS I or increase of the severity of CRPS I symptoms in the affected limb, or the risk of inducing CRPS I in a healthy limb (Veldman et al., 1993). We had the opportunity to study the MEGS in muscle tissue of end-stage CRPS I patients requiring amputation of the affected limb. MEGS parameters were compared to those observed in previously published control muscle samples (Janssen et al., 2006) and in an additional control group consisting of patients with peripheral arterial occlusive disease (PAOD) undergoing amputation of a limb due to end-stage of the disease. The PAOD patients showed hypoxia and necrosis in the affected limb without CRPS I characteristics.

2. Methods

2.1. Patients

Eight patients (seven females, one male) with severe, chronic CRPS I of an extremity, fulfilling the diagnostic criteria of the IASP and Veldman et al. Mersky and Bogduk (1994), Stanton-Hicks et al. (1995) were included in this study. Patients had received extensive treatment with sympathetic blockades, free radical scavengers, occupational therapy and pain-relieving drugs, following a protocol developed by our group (Tan et al., 2010; van der Laan and Goris, 1997). Amputation of the affected extremity was necessary because CRPS I had resulted in a non-functional limb with severe hyperalgesia (Table 1). The indication for amputation was previously documented in a large study of patients with CRPS I (Dielissen et al., 1995). The study was approved by the University Hospital Ethical Committee and was performed in adherence with the principles of the Declaration of Helsinki. Informed consent was obtained to acquire blood samples and muscle tissue of the amputated extremities to perform the biochemical studies. To evaluate impaired oxygen metabolism, blood gas samples were obtained at the beginning of the operation, after visualizing the superficial femoral vein and artery, and before clamping, and promptly analyzed with the Bayer Rapidlab 865 (Siemens Medical Solutions, Breda, The Netherlands). No blood gas control samples were taken from the contra-lateral extremity, considering the risk of inducing CRPS I in the unaffected extremity. The mean age at the time of the amputation was 38.5 years (range 20–60 years). The mean interval between injury and onset of CRPS I symptoms was 2 days (range 0–31 weeks). The mean duration of CRPS I symptoms preceding the amputation was 9 years (range 1–24 years). In 75% of cases, CRPS I was induced by a minor injury, a surgical procedure, a contusion or sprain. Two patients were smokers and one patient still used vasodilators as treatment for CRPS I. None of the patients had migraine, were diabetic, had gastro-intestinal dysmotility problems, anxiety, peripheral vascular disease, or showed symptoms of central or peripheral nervous system disease.

2.2. Controls

MEGS parameters were compared to those observed in previously published control muscle samples (age 2–59 years) Janssen et al., 2006. There are no differences in MEGS parameters between M. semitendinosus, M. quadriceps, and M. gastrocnemius (Janssen et al., 2006) and unpublished observations). An additional control group for MEGS parameters and blood gas analysis consisted of patients with peripheral arterial occlusive disease (PAOD) that underwent amputation of an extremity due to end-stage PAOD (for details see Table 1). Although there was no perfect match for gender and age between the CRPS I patient group and the PAOD patient control group, both groups may be mutually compared because in the healthy control group we found no significant correlation between age and MEGS parameters, age and respiratory chain enzyme activities and no significant differences for gender for these parameters (Janssen et al., 2006). Patients matched for age and gender, undergoing elective surgery with general anesthesia (removal of osteosynthesis material, laparoscopic cholecystectomy, excision of breast lump and treatment of anal fistula) were used as controls for blood gas analysis. Immediately after induction of anesthesia and before incision, blood samples (2 mL) were taken from the femoral vein and promptly analyzed with a Bayer Rapidlab 865 analyzer for pO_2, pH, lactate, glucose, and venous oxygen saturation (SvO_2).

2.3. Biochemical and molecular genetic studies

The MEGS encompasses the whole chain of mitochondrial enzymatic reactions involved in the generation of ATP, including: (I) transport of pyruvate and other substrates into the mitochondrion, (II) oxidation of pyruvate by the pyruvate dehydrogenase complex to acetyl-CoA, NADH and CO_2 , (III) oxidation of acetyl-CoA to CO_2 in the tricarboxylic acid cycle to yield NADH and $FADH_2$, (IV) oxidation of NADH and $FADH_2$ in the respiratory chain with subsequent coupled ATP production by complex V, and (V) transport of ATP out of the mitochondrion by the adenine nucleotide translocator. Immediately after amputation of the affected limb, fresh muscle specimens (M. gastrocnemius of seven CRPS I patients and six PAOD patients, and the M. extensor digitorum of one CRPS I patient) were obtained and put in an ice-cold sample buffer as described before (Janssen et al., 2006). Within 1 h, mitochondrial enriched fractions were prepared and the MEGS capacity was investigated by measuring the $^{14}CO_2$ production rate from oxidation of [1-^{14}C] pyruvate in the presence of malate and ADP, and by measuring the ATP production rate from oxidation of pyruvate in the presence of malate and ADP. These two parameters are a measure for the overall MEGS capacity, and any disturbance of the MEGS will lead to lower pyruvate oxidation and ATP production rates. The oxidation rate of [U-^{14}C] malate was measured as a measure for MEGS capacity and citric acid cycle activity. The oxidation rate of [1,4-^{14}C] succinate was measured as a measure for complex II activity in fully intact mitochondria. Detailed information about these methods is described in Janssen et al. (2006). In two CRPS I patients, a fresh sample could not be obtained and instead the muscle sample was snap frozen in liquid nitrogen immediately after collection. Mitochondria-enriched fractions were prepared essentially following the same procedure as for fresh muscle samples. As frozen samples are not suitable for analysis of substrate oxidations or ATP production, only respiratory chain enzyme activities were measured in these samples. Respiratory chain complex I in control and CRPS I muscle was determined by spectrophotometric measurements of the rotenone-sensitive NADH oxidation at 340 nm with coenzyme Q_1 as an artificial electron acceptor (Fischer et al., 1986). As this assay is no longer operational in our lab, complex I in PAOD muscle was determined by spectrophotometric measurements at 600 nm of the rotenone sensitive reduction of 2,6-dichloroindophenol from oxidation of NADH via an intermediate electron acceptor, decylubiquinone (Janssen et al., 2007). In order to compare complex I activities between different groups of patients/controls, a relative complex I activity was calculated as a percentage of the mean of each group. Complex II activity was measured by spectrophotometric analysis of the reduction of 2,6-dichloroindophenol from oxidation of succinate at 600 nm (Janssen et al., 2007). Complex III activity was determined by spectrophotometric measurements of the reduction of cytochrome c from the oxidation of decylubiquinone at 550 nm (Zheng et al., 1990). Complex IV was measured by spectrophotometric analysis of the oxidation cytochrome c at 550 nm (Cooperstein and Lazarow, 1951). Citrate synthase was analyzed by

Please cite this article in press as: Tan ECT et al. Mitochondrial dysfunction in muscle tissue of complex regional pain syndrome type I patients. Eur J Pain (2011), doi:10.1016/j.ejpain.2010.12.003

Table 1
S_vO_2 in eight patients with therapy-resistant severe chronic CRPS I and six patients with peripheral arterial occlusive disease (PAOD).

Patient	Gender	Age at amputation (years)	Inciting event	Limb	Past medical history	Duration CRPS I prior to amputation (years)	Most important indication for amputation	Level of amputation	Treatment prior to amputation	S_vO_2 (%)
CRPS										
1	F	38	Sprained ankle	Leg	None	4	Persistent infection, severe oedema, no function	TK	OFRS, VD, TENS, SB, ST	85
2	F	34	Minor injury	Leg	Arthroscopy knee	3	No function, extreme equinovarus	AK	OFRS, VD	91
3	F	60	Spontaneous	Leg	Uterus resection	21	Persistent infection, no function	AK	OFRS, VD, SCS	92
4	F	57	Spontaneous	Arm	COPD, MI	1	No function, clenched fist	TE	OFRS, PT, VD, OT, SB	93
5	M	34	Sprained ankle	Leg	Arthroscopy knee	4	No function, severe oedema	AK	OFRS, VD, TENS, SCS	97
6	F	41	Car accident	Leg	None	24	No function	TK	PT, OFRS, VD, TENS, Psy, SB, ST	99
7[a]	F	24	Foot injury playing soccer	Leg	Triple arthrodesis foot	10	No function, chronic ulceration	TK	PT, OFRS, VD	94
8[a]	F	20	Sprained ankle	Leg	ADHD, ovarial abcess	5	Oedema, chronic ulceration, no function	AK	OFRS, VD, SB	95
PAOD										
1	M	62	na	Leg	pT_3N_0 squamous cell carcinoma tongue, DM, MI, HT, HC	2	PAOD with tissue loss	BK	PTA, several stents and venous bypass operations	59
2	M	78	na	Leg	$pT_3N_2M_0$ prostate carcinoma, HT, HC, MI, adipositas	12	PAOD with tissue loss	AK	Stents and aorto bifemoral prosthesis, venous bypass operations	nd
3	M	80	na	Leg	COPD, HF	1	PAOD with tissue loss	AK	PTA, several stents	70
4	M	46	na	Leg	DM, alcohol abuse	4	Diabetic foot and PAOD with tissue loss	BK	Wound care, ambulation	52
5	M	71	na	Leg	Mental retardation, MI, sick sinus syndrome	3	PAOD with tissue loss	AK	Femoro-femoral crossover bypass, PTA	88
6	M	76	na	Leg	HT, TIA, HF	1	PAOD with tissue loss	BK	Wound care	91

CRPS I, complex regional pain syndrome type I; PAOD, peripheral arterial occlusive disease. F, female; M, male. na, not applicable. DM, diabetes mellitus; HT, hypertension; HC, hypercholesterolemia; COPD, chronic pulmonary obstructive disease; MI, myocardial infarction; ADHD, attention deficit hyperactivity disorder; HF, heart failure; TIA, transient ischemic attack. TK, through knee; AK, above knee; TE, through elbow; AE, above elbow. PT, physiotherapy; OFRS, oxygen free radical scavengers; VD, various drugs; TENS, transcutaneous electrical nerve stimulation; SCS, spinal cord stimulation; OT, occupational therapy; Psy, psychological treatment; SB, sympathetic block; ST, sympathectomy; PTA, percutaneous transluminal angioplasty. nd, not determined.

[a] Patients with snap frozen muscle sample.

Table 2
Biochemical analysis of the MEGS in muscle tissue of CRPS I and AOD patients.

Patient	Muscle investigated	pyr + mal[a]	mal + pyr[a]	suc + acc[a]	ATP[a]
CRPS					
1	*M. gastrocnemius*	5.13	6.61	4.08	52.7
2	*M. gastrocnemius*	0.87	1.20	nd	10.6
3	*M. gastrocnemius*	5.17	5.58	3.46	44.7
4	*M. extensor digitorum*	3.91	4.77	2.88	46.6
5	*M. gastrocnemius*	1.64	1.46	1.37	1.9
6	*M. gastrocnemius*	2.51	2.95	1.89	23.6
7	*M. gastrocnemius*	nd[b]	nd[b]	nd[b]	nd[b]
8	*M. gastrocnemius*	nd[b]	nd[b]	nd[b]	nd[b]
AOD					
1	*M. gastrocnemius*	7.81	6.57	4.78	53.2
2	*M. gastrocnemius*	4.62	4.07	3.61	48.5
3	*M. gastrocnemius*	3.67	3.90	3.56	37.3
4	*M. gastrocnemius*	6.46	6.82	4.26	62.8
5	*M. gastrocnemius*	7.78	6.19	3.54	39.5
6	*M. gastrocnemius*	4.99	4.53	2.25	41.8
	Controls (observed range)	3.45–7.99	3.28–8.80	2.03–4.18	36.0–81.7
	Controls range (mean ± 2sd)	3.51–8.01	3.78–8.96	2.04–4.26	36.1–76.8
	n	42	41	33	40
	p MW U test: controls versus CRPS	0.002	0.005	0.517	0.002
	p MW U test: controls versus AOD	0.963	0.104	0.08	0.047
	p MW U test: AOD versus CRPS	0.078	0.262	0.1	0.2

[a] Substrate oxidation rates and ATP production rates are given in nmol $^{14}CO_2$ or ATP/h mU citrate synthase (CS), respectively.
[b] As in patients 7 and 8 a frozen muscle sample was examined, ATP production and substrate oxidation rates could not be measured. nd: not determined.

spectrophotometric measurements of the reduction of dithionitrobenzoic acid at 412 nm by coenzyme A, the product of this enzyme (Srere, 1969). The citrate synthase activity was used as a mitochondrial reference to normalize all other mitochondrial parameters. Protein was measured by the method of Lowry et al. (1951). The complete mitochondrial genome was studied in muscle tissue of the CRPS I patients with the Affymetrix Gene Chip® Human Mitochondrial Resequencing Array 2.0, according to the manufacturer's instructions with minor modifications (www.affymetrix.com).

2.4. Immunochemical studies on muscle mitochondria

Mitochondrial fractions were isolated using a differential centrifugation procedure as described (Janssen et al., 2007). Isolated mitochondrial fractions were subjected to immunoblotting for carbonylated proteins, manganese superoxide dismutase (MnSOD) and porin. Protein carbonylation, as a measure of oxidative damage, was studied using the Oxyblot™ Oxidized Protein Detection Kit (Chemicon) in isolated muscle mitochondrial fractions according to the manufacturer's instructions with minor modifications (http://www.Millipore.com/catalogue/item/s7150#). IRDye™ 680 conjugated goat-anti-rabbit IgG (Licor) was used as second antibody. MnSOD was studied with rabbit-anti-MnSOD (Stressgen) as first antibody and IRDye™ 800 conjugated goat-anti-rabbit IgG (Licor) as second antibody. Porin was used to normalize for the amount of mitochondrial protein in each lane. For detection of this protein Mouse Anti-porin 31-HL (Ab-3) (Calbiochem) was used as primary antibody. IRDye™ 800 or IRDye™ 680 conjugated goat-anti-mouse IgG (Licor) were used as second antibodies for the Oxyblot and the MnSOD blot, respectively. The blots were scanned on a Licor Odyssey scanner. Missing data due to limited availability of muscle sample are MnSOD in CRPS I patients 4 and 5 and oxyblot in CRPS I patients 4 and 6.

2.5. Statistics

Biochemical data of the MEGS and the respiratory chain enzymes of the CRPS I patients, the PAOD patients and 42 control

Table 3
Biochemical analysis of the respiratory chain enzymes in muscle tissue of CRPS I and AOD patients.

Patient	Muscle investigated	Complex I[d]	Complex II[b]	Complex III[b]	Complex IV[b]	CS[c]
CRPS						
1	*M. gastrocnemius*	107	566	3322	1674	171
2	*M. gastrocnemius*	85	545	2242	1597	26
3	*M. gastrocnemius*	64	457	3047	1078	105
4	*M. extensor digitorum*	97	627	2738	1471	43
5	*M. gastrocnemius*	57	357	1942	1339	46
6	*M. gastrocnemius*	73	441	2050	1449	28
AOD						
1	*M. gastrocnemius*	83	577	3331	1517	38
2	*M. gastrocnemius*	88	844	3610	1252	17
3	*M. gastrocnemius*	98	741	3154	949	86
4	*M. gastrocnemius*	63	737	3376	1081	70
5	*M. gastrocnemius*	77	680	2867	1839	72
6	*M. gastrocnemius*	67	840	3518	1341	55
	Controls (observed range)[a]	62–192	333–853	2057–4964	1201–2938	23–178
	Controls range (mean ± 2sd)[a]	53–147	359–884	1899–4569	1099–2563	22–168
	n	41	35	35	42	42
	p MW U test: controls versus CRPS	0.045	0.024	0.034	0.076	0.115
	p MW U test: controls versus AOD	0.031	0.036	0.555	0.021	0.016
	p MW U test: AOD versus CRPS	1.0	0.006	0.016	0.522	1.0
CRPS						
7	*M. gastrocnemius*	65	nd	1192	878	nd
8	*M. gastrocnemius*	108	nd	1289	1088	nd
	Controls (observed range)[a]	101–389	nd	1020–2530	520–2080	nd

nd: not determined.
[a] Samples from CRPS I patients 1–6 and from the AOD patients were "fresh" muscle biopsies, whereas for CRPS I patients #7 and #8 the biopsies were frozen after collection. Fresh and frozen samples were processed in a different manner and therefore, different control values apply.
[b] Complexes II–IV activity in fresh muscle tissue is given in mU/U CS, complex I and III activity in frozen muscle tissue in mU/U complex IV, and complex IV activity in frozen muscle tissue in mU/U CS.
[c] CS activity is given in mU/mg protein.
[d] Complex I activity is given as a relative activity as described in the Section 2.

Please cite this article in press as: Tan ECT et al. Mitochondrial dysfunction in muscle tissue of complex regional pain syndrome type I patients. Eur J Pain (2011), doi:10.1016/j.ejpain.2010.12.003

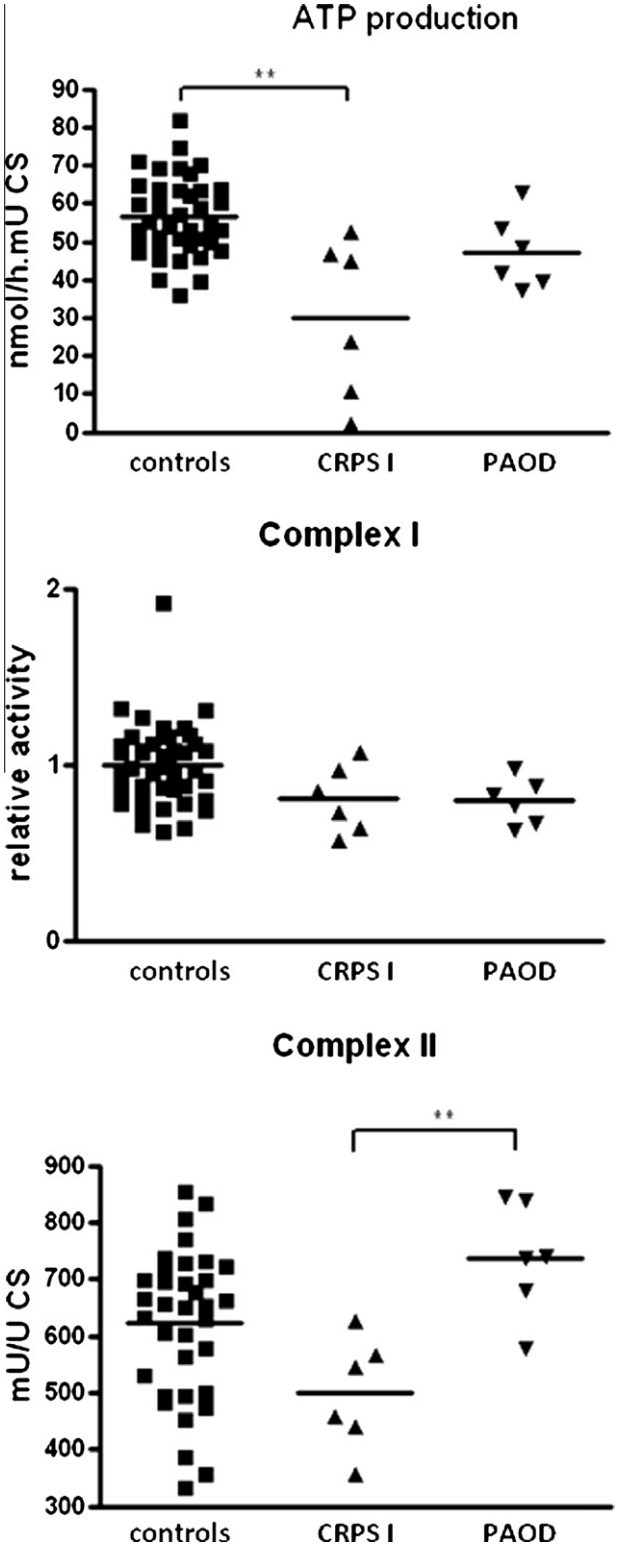

Fig. 1. ATP production rates, complex I and complex II activities in muscle of controls, CRPS I patients and PAOD patients. Dot plots for the ATP production rates, complex I activity and complex II activity. ATP production is expressed in nmol ATP/h.mU citrate synthase, complex I as a relative activity (see Section 2), and complex II as mU/U citrate synthase. ■ Controls, ▲ CRPS I patients, ▼ PAOD patients. $^{**}p \leqslant 0.01$ in the Mann Whitney U test.

individuals (Janssen et al., 2006) were compared using the non-parametric Mann–Whitney U test (MW U test). Differences were considered to be statistically significant if $p < 0.01$. MEGS parameters and respiratory chain enzyme activities were compared to a reference range based on the mean ± 2SD of values obtained in healthy control samples ($n = 43$), as described before (Janssen et al., 2006).

3. Results

Table 1 gives the venous oxygen saturation values measured in blood of the affected limb of CRPS I and PAOD patients just prior to the amputation, showing that the SvO_2 values were increased in all CRPS I patients and in two PAOD patients. The SvO_2 values were decreased in three other PAOD patients. The MEGS parameters (oxidation rates of [1-^{14}C] pyruvate + malate + ADP, [U-^{14}C] malate + pyruvate + ADP, and the ATP production rate from oxidation of pyruvate) were significantly lower in the CRPS I group compared to the control group, and did not significantly differ between the PAOD patients and controls (Table 2). Although all values of the MEGS parameters measured in the PAOD patients were within the control range, we found no statistically significant difference between the CRPS I and the PAOD group. All MEGS parameters were below the reference range in three out of six CRPS I patients. The oxidation rate of [1,4-^{14}C] succinate + acetylcarnitine was significantly decreased in two of five patients. We found no significant differences for the respiratory chain enzymes complex I, III and IV between the three groups (Table 3). Complex II activity was significantly increased in the PAOD group compared to the CRPS I group (Table 3). All respiratory chain enzyme activities were normal in the CRPS I and the PAOD patients, except for the activity of complex IV that was slightly below the reference range in one CRPS I and two PAOD patients. In one of two CRPS I patients we found a decreased activity of complex I in frozen muscle tissue. Typical dot plots for the MEGS parameters (ATP production rate) and respiratory chain enzymes (complex I and II activity) are presented in Fig. 1. In search for evidence of elevated ROS, we tested for the presence of carbonylation of mitochondrial proteins that is indicative of oxidative damage. In all CRPS I patients we found a varying degree of increase in oxidative damage to mitochondrial proteins (Fig. 2A). In patients #2 and #7 a strong increase in oxidative damage to muscle proteins was observed. In patients #5, #1, #3 and #8, there was an increased amount of oxidative damage to proteins with a molecular weight of approximately 250 kDa, while in proteins of lower molecular weight the level of oxidative protein damage appeared to be similar to controls. Although the nature of these carbonylated proteins is as yet unknown, these results clearly show an increase in proteins with oxidative damage in muscle tissue of CRPS I patients. In patients #2 and #7 showing the highest amounts of carbonylated proteins, we found a decreased level of MnSOD (Fig. 2A). Patient #2 also showed the strongest decrease in the MEGS parameters (Table 2). In most PAOD patients, protein carbonylation was similar to that seen in control muscle, except for the muscle sample of PAOD patient #2 which appeared to have a slight, relative increase in protein carbonylation. The amount of MnSOD was more or less increased in five PAOD patients while it was normal in patient #2 (Fig. 2B).

4. Discussion

In this study we found evidence for mitochondrial dysfunction and ROS pathology in muscle tissue from CRPS I patients. The MEGS activity in muscle and other tissues is dependent on normal oxygen availability, as oxygen is the final electron acceptor of the mitochondrial respiratory chain. Under hypoxic conditions, both the oxidation rate of pyruvate and the ATP production rate are decreased due to a decreased oxidation of NADH and $FADH_2$ by the respiratory chain. Under these conditions, pyruvate will be

Please cite this article in press as: Tan ECT et al. Mitochondrial dysfunction in muscle tissue of complex regional pain syndrome type I patients. Eur J Pain (2011), doi:10.1016/j.ejpain.2010.12.003

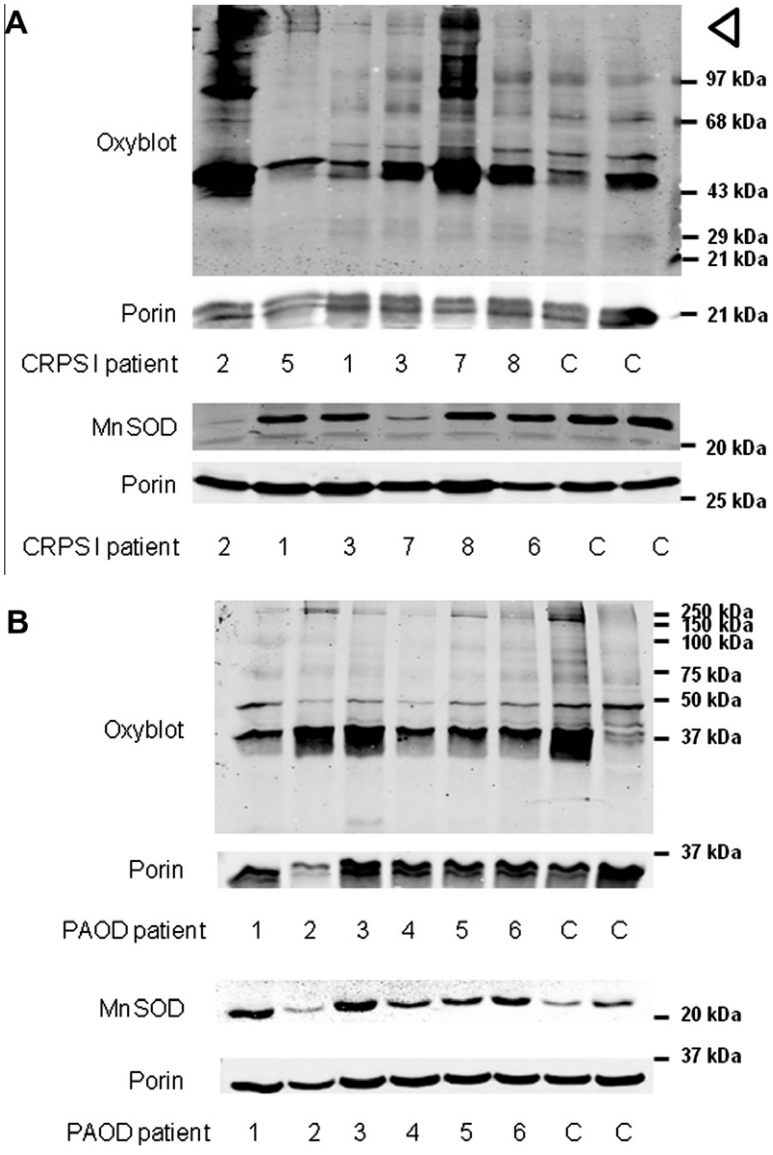

Fig. 2. Carbonylation of mitochondrial proteins and MnSOD levels in muscle mitochondria from CRPS I patients and PAOD patients. Patient numbering is the same as in Table 1; healthy control samples are indicated by "C". Protein carbonylation as a measure for oxidative damage was determined by Oxyblot (for details see Section 2) and MnSOD levels were determined by Western blot. (A) Observations in CRPS I muscle. Patients #2 and #7 show an increased amount of carbonylated proteins (top panel) and reduced MnSOD levels (bottom panel). The arrowhead indicates the position of increased oxidative damage to proteins in patients #5, #1, #3, and #8, which were also strongly increased in patients #2 and #7. The immunoblot for porin from the same gel is presented below each single blot, and indicates equal loading of proteins. Molecular weights indicated on the right are based on a molecular weight marker run on the same gels. (B) Observations in PAOD muscle. Patient #2 shows an increased amount of oxidative damage to proteins in relation to the amount of the mitochondrial marker protein porin (top panel) and increased amounts of MnSOD in patients #1, #3, #4, #5 and #6 (bottom panel). The immunoblot for porin from the same gel is presented below each single blot. Molecular weights indicated on the left are based on a molecular weight marker run on the same gels.

converted to lactic acid, resulting in lactic acidosis. During chronic and extreme hypoxic conditions, muscle tissue fails to maintain a normal redox state. This results in an increased ROS production, followed by cell injury or dysfunction (Clanton, 2007). In end-stage CRPS I patients we have observed significantly increased venous oxygen saturation values at the level of amputation of the affected limb just prior to the surgical procedure. In a previous study in the affected limbs of 25 CRPS I patients we have found a mean venous oxygen saturation level of 94.3 ± 4.0%, which is significantly higher than the levels of 77.5 ± 9.8% observed in healthy subjects.(Tan et al., 2010). This is indicative of either a decreased oxygen diffusion, leading to mitochondrial dysfunction, or utilization, caused by mitochondrial dysfunction within the affected limb, or a combination of both (Goris, 1998). Previously, mitochondrial dysfunction has been observed in P NMR spectroscopy studies of CRPS I patients, which revealed a disturbed phosphate/phosphocreatine metabolism in skeletal muscle of the affected limb (Heerschap et al., 1993). In the present study we observed a reduced MEGS capacity in muscle tissue of CRPS I patients. In a second group of patients, suffering from PAOD, we found that the venous oxygen saturation values were either increased or decreased, which indicates that also in these patients oxygen metabolism is disturbed in the affected limb. However, in this group of patients all the MEGS parameters showed normal activities, while complex II activity in the CRPS I patients was significantly lower than in the PAOD patients. These observations indicate that the response of mitochondrial energy metabolism to hypoxic conditions in CRPS I patients differs from that in PAOD patients. From recent studies it is known that an impaired tricarboxylic acid cycle flux, particularly if it is caused by decreased activity of complex II, might result

Please cite this article in press as: Tan ECT et al. Mitochondrial dysfunction in muscle tissue of complex regional pain syndrome type I patients. Eur J Pain (2011), doi:10.1016/j.ejpain.2010.12.003

in decreased mitochondrial energy production and in overproduction of free radicals. Accumulation of succinate subsequently leads to inhibition of prolyl hydroxylases, thereby stabilizing hypoxia-inducible factors (HIFs). These HIFs bind to hypoxia-responsive elements so activating the transcription of more than two hundred genes that allow cells to adapt to the hypoxic condition (Solaini et al., 2010). As mentioned before, mitochondrial dysfunction caused by a decreased oxygen availability will lead to an increase of ROS, resulting in carbonylated mitochondrial proteins, which is indicative of oxidative damage (Choksi et al., 2004). In all CRPS I patients and in one out of six PAOD patients, we found a varying degree of increase in oxidative damage to mitochondrial proteins, while the amount of MnSOD, a mitochondrial antioxidant, was decreased in two CRPS I patients and more or less increased in five PAOD patients. This indicates that ROS pathophysiology in PAOD muscle differs from that in CRPS I muscle. Our observations are compatible with the involvement of free radicals in the pathophysiology of CRPS I that has been reported previously (Eisenberg et al., 2008; Perez et al., 2003; van der Laan et al., 1998; van der Laan and Goris, 1997). An increase in lipid peroxidation products in serum and increased antioxidative products has been observed in serum and saliva of CRPS I patients (Eisenberg et al., 2008). These patients showed increased SOD activity in saliva, which in all probability is Cu/Zn-SOD, instead of the mitochondrial MnSOD studied here. Little is known about the role of genetic factors in CRPS I. Recently, Higashimoto et al. described eight children with mitochondrial disease and clinical features meeting the IASP diagnostic criteria for CRPS (Higashimoto et al., 2008). In two of them decreased respiratory chain complex activities were measured and in six of them the clinical features and family history pointed towards a possible mtDNA mutation. In four of their patients mtDNA transitions were detected, all known as polymorphisms in the mtDB database (Ingman and Gyllensten, 2006). Whether these polymorphisms are risk factors for developing CRPS is unknown. In our patients we performed sequence analysis of the entire mtDNA but found no pathogenic mtDNA mutations (data not shown).

This study was performed in a relatively small number of CRPS I patients at the end-stage of their disease and should be regarded as a pilot study providing for the first time biochemical evidence for a role of mitochondria in CRPS I. As this is the first study to explore the relationship between mitochondrial energy metabolism and CRPS I, no particular strength of association between CRPS I and individual MEGS parameters was suspected. Therefore we investigated the association between all potentially relevant parameters and did not apply a post hoc correction for multiple testing. This study does not answer the question if the mitochondrial dysfunction is a primary event in the pathophysiology of CRPS I, or is secondary to CRPS I. Our study shows a correlation between mitochondrial energy metabolism, ROS-induced protein damage, and CRPS I, but additional studies are required to clarify the causal relationships between these features. To gain further insight in this matter, it would be interesting to examine muscle mitochondrial function in earlier stages of the disease, and also in an unaffected limb of the same patient. However, as a muscle biopsy holds the risk of inducing, or increasing the severity of, CRPS I (Veldman et al., 1993), less invasive procedures to test mitochondrial function are preferred, such as screening for metabolites in blood and urine. It should be noted that the biochemical examination of a muscle biopsy is generally regarded as the golden standard to examine mitochondrial function, and that other diagnostic tests have a much greater chance of false negative results (Haas et al., 2008; Rodenburg, 2010). Finally, it is important to note that amputation of affected limbs should not be regarded as standard treatment for CRPS I patients, and should only be considered for patients that have been resistant to all forms of therapy and are in the end-stage of the disease where they risk encountering complications such as severe infections, trophic ulcers and intractable pain. The indications for amputation, which is only rarely considered, have been documented in a report on a large cohort of CRPS I patients (Dielissen et al., 1995).

In summary, our study is the first to reveal mitochondrial dysfunction and ROS pathology in muscle tissue from CRPS I patients. At this moment, it is unclear whether the mitochondrial dysfunction that is apparent at the end-stage of CRPS I could also be present in earlier stages of the disease, or are secondary to CRPS I, and further studies are needed to obtain an answer to this question. The observations in muscle from PAOD patients indicate that the mitochondrial dysfunction seen in CRPS I is not a general phenomenon of severe, hypoxic muscle disease. On the basis of our findings we postulate that an impaired mitochondrial energy metabolism is involved in the pathogenesis of CRPS I.

Acknowledgments

The authors wish to thank the technicians of the muscle lab of the Laboratory of Genetic, Endocrine, and Metabolic Disorders, Department of Laboratory Medicine, for excellent technical assistance. Part of this work was supported by the Energy4All foundation (RR). The authors declare no conflicts of interest.

References

Choksi KB, Boylston WH, Rabek JP, Widger WR, Papaconstantinou J. Oxidatively damaged proteins of heart mitochondrial electron transport complexes. Biochim Biophys Acta 2004;1688:95–101.

Clanton TL. Hypoxia-induced reactive oxygen species formation in skeletal muscle. J Appl Physiol 2007;102:2379–88.

Cooperstein SJ, Lazarow A. A microspectrophotometric method for the determination of cytochrome oxidase. J Biol Chem 1951;189:665–70.

De Mos M, de Bruijn AG, Huygen FJ, Dieleman JP, Stricker BH, Sturkenboom MC. The incidence of complex regional pain syndrome: a population-based study. Pain 2007;129:12–20.

Dielissen PW, Claassen AT, Veldman PH, Goris RJ. Amputation for reflex sympathetic dystrophy. J Bone Joint Surg Br 1995;77:270–3.

Eisenberg E, Shtahl S, Geller R, Reznick AZ, Sharf O, Ravbinovich M, et al. Serum and salivary oxidative analysis in complex regional pain syndrome. Pain 2008;138:226–32.

Fischer JC, Ruitenbeek W, Trijbels JM, Veerkamp JH, Stadhouders AM, Sengers RC, et al. Estimation of NADH oxidation in human skeletal muscle mitochondria. Clin Chim Acta 1986;155:263–73.

Goris RJ. Reflex sympathetic dystrophy: model of a severe regional inflammatory response syndrome. World J Surg 1998;22:197–202.

Haas RH, Parikh S, Falk MJ, Saneto RP, Wolf NI, Darin N, et al. The in-depth evaluation of suspected mitochondrial disease. Mol Genet Metab 2008;94:16–37.

Heerschap A, den Hollander JA, Reynen H, Goris RJ. Metabolic changes in reflex sympathetic dystrophy: a 31P NMR spectroscopy study. Muscle Nerve 1993;16:367–73.

Higashimoto T, Baldwin EE, Gold JI, Boles RG. Reflex sympathetic dystrophy: complex regional pain syndrome type I in children with mitochondrial disease and maternal inheritance. Arch Dis Child 2008;93:390–7.

Ingman M, Gyllensten U. mtDB: Human Mitochondrial Genome Database, a resource for population genetics and medical sciences. Nucleic Acids Res 2006;34:D749–51.

Janssen AJ, Trijbels FJ, Sengers RC, Smeitink JA, van den Heuvel LP, Wintjes LT, et al. Spectrophotometric assay for complex I of the respiratory chain in tissue samples and cultured fibroblasts. Clin Chem 2007;53:729–34.

Janssen AJ, Trijbels FJ, Sengers RC, Wintjes LT, Ruitenbeek W, Smeitink JA, et al. Measurement of the energy-generating capacity of human muscle mitochondria: diagnostic procedure and application to human pathology. Clin Chem 2006;52:860–71.

Klimova T, Chandel NS. Mitochondrial complex III regulates hypoxic activation of HIF. Cell Death Differ 2008;15:660–6.

Lowry O, Rosebrough N, Farr A, Randall R. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.

Mersky H, Bogduk N, editors. Classification of chronic pain: description of chronic pain syndromes and definitions of terms. Seattle: IASP Press; 1994.

Perez RS, Zuurmond WW, Bezemer PD, Kuik DJ, van Loenen AC, de Lange JJ, et al. The treatment of complex regional pain syndrome type I with free radical scavengers: a randomized controlled study. Pain 2003;102:297–307.

Robinson JN, Sandom J, Chapman PT. Efficacy of pamidronate in complex regional pain syndrome type I. Pain Med 2004;5:276–80.

Rodenburg RJ. Biochemical diagnosis of mitochondrial disorders. J Inherit Metab Dis 2010 [epub, PMID: 20440652].

Please cite this article in press as: Tan ECT et al. Mitochondrial dysfunction in muscle tissue of complex regional pain syndrome type I patients. Eur J Pain (2011), doi:10.1016/j.ejpain.2010.12.003

Solaini G, Baracca A, Lenaz G, Sgarbi G. Hypoxia and mitochondrial oxidative metabolism. Biochim Biophys Acta 2010;1797:1171–7.

Srere PA. Citrate synthase, EC 4.1.3.7, citrate oxaloacetate lyase (CoA-acetylating). Methods Enzymol 1969;13:3–11.

Stanton-Hicks M, Janig W, Hassenbusch S, Haddox JD, Boas R, Wilson P. Reflex sympathetic dystrophy: changing concepts and taxonomy. Pain 1995;63:127–33.

Tan EC, Ter Laak HJ, Hopman MT, van GoorH, Goris RJA. Impaired oxygen utilization in skeletal muscle of CRPS I patients. J Surg Res 2010.

Tran de QH, Duong S, Bertini P, Finlayson RJ. Treatment of complex regional pain syndrome: a review of the evidence. Can J Anaesth 2010;57:149–66.

van der Laan L, Goris RJ. Reflex sympathetic dystrophy. An exaggerated regional inflammatory response? Hand Clin 1997;13:373–85.

van der Laan L, Ter Laak HJ, Gabreels-Festen A, Gabreels F, Goris RJ. Complex regional pain syndrome type I (RSD): pathology of skeletal muscle and peripheral nerve. Neurology 1998;51:20–5.

Veldman PH, Reynen HM, Arntz IE, Goris RJ. Signs and symptoms of reflex sympathetic dystrophy: prospective study of 829 patients. Lancet 1993;342:1012–6.

Zheng XX, Shoffner JM, Voljavec AS, Wallace DC. Evaluation of procedures for assaying oxidative phosphorylation enzyme activities in mitochondrial myopathy muscle biopsies. Biochim Biophys Acta 1990;1019:1–10.

Please cite this article in press as: Tan ECT et al. Mitochondrial dysfunction in muscle tissue of complex regional pain syndrome type I patients. Eur J Pain (2011), doi:10.1016/j.ejpain.2010.12.003