14. Unusual clinical features associated with FSHD

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14.1 Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is a dominantly inherited myopathy usually associated with a deletion of 3.3 kb *Kpn*I repeated units (D4Z4) on chromosome 4q35 (FSHMD1A; MIM 158900).

Typical clinical symptoms are characterized by unique involvement of muscles, which usually progress in a descending manner, including weakness and atrophy of facial muscles, followed by the shoulder girdle, the scapula fixators, and the upper arm muscles. Subsequently, pelvic girdle and lower limbs are also involved, and eventually, some 20% of the patients become wheelchair-bound by the age of 40 years (Lunt and Harper, 1991). Difficulties in whistling, closing the eyes, or lifting arms overhead are common initial symptoms. Prominent scapular winging and horizontally positioned clavicles are also observed. Facial or shoulder girdle weakness usually appears in adolescence, but signs may be apparent on examination in early childhood. Asymmetry of muscle involvement is often observed in apparently affected patients, but not related to handedness (Tawil *et al.*, 1994). Weakness is relatively mild and the progression is usually quite slow.

The clinical diagnosis of FSHD is sometimes difficult because the onset of the disease and the phenotypic expression is extremely variable, both within and between families (Lunt *et al.*, 1995; Padberg *et al.*, 1995b). One family may show severe disabilities with involvement of organs other than skeletal muscles, whereas others remain almost asymptomatic. Recent reports have shown much broader clinical expression of FSHD than perhaps previously recognized. In this chapter, unusual clinical features of FSHD are reviewed.

14.2 Early-onset form of FSHD

FSHD is generally a benign, slowly progressive myopathy that begins in late childhood or adolescence, and leads to disability only late in its course. However, some

patients exhibit clinical symptoms from infancy or early childhood.

Korf *et al.* (1985) reported six patients in whom facial diplegia occurred in the first year of life, with subsequent development of facioscapulohumeral dystrophy. All had severe progressive disability prior to adolescence. Facial involvement did not include extraocular muscles. All six patients had a sensorineural hearing loss (Korf *et al.*, 1985). Bailey *et al.* (1986) also reported clinical, electrodiagnostic, and biopsy findings in a family with infantile FSHD. Four of eight family members having the disorder, all with onset in infancy, developed severe weakness leading to death in adolescence (Bailey *et al.*, 1986).

Although these reports have suggested that progressive and severe infantile FSHD is a genetically different form of FSHD, Brouwer *et al.* (1994) proposed them to be part of a wide clinical spectrum of FSHD. They designated the criteria for the early-onset form of FSHD as follows: (1) signs or symptoms of facial weakness before the age of 5 years; and (2) signs or symptoms of shoulder girdle weakness before the age of 10 years (Brouwer *et al.*, 1994).

Clinical features of the early-onset patients are similar but more severe, progressive, and variable (Kilmer *et al.*, 1995; Funakoshi *et al.*, 1998; Yamanaka *et al.*, 2002). Patients present with early-onset facial weakness or diplegia (Shapiro *et al.*, 1991; Jardine *et al.*, 1994a). Gait disturbances are observed before 28 years of age, and significantly earlier than the other group (Shapiro *et al.*, 1991; Jardine *et al.*, 1994a; Yamanaka *et al.*, 2002). Furthermore, the early-onset patients are often accompanied by bilateral sensorineural hearing loss, retinal vasculopathy, mental retardation and epilepsy (Shapiro *et al.*, 1991; Brouwer *et al.*, 1994; Funakoshi *et al.*, 1998; Miura *et al.*, 1998; Yamanaka *et al.*, 2002).

Nakagawa *et al.* (1997) detected the early-onset form in 17% of Japanese FSHMD1A patients. Yamanaka *et al.* (2002) estimated the frequency of early onset type of FSHD to be 13.4% (31/231 Japanese FSHMD1A patients from 145 unrelated families), and that was seen more frequently in sporadic cases. Genetic analysis revealed that they had significantly larger gene deletions on chromosome 4q35, and the patients with the smallest size of *Eco*RI fragment (10–11 kb) were usually of the early-onset type (Kilmer *et al.*, 1995; Funakoshi *et al.*, 1998; Yamanaka *et al.*, 2002).

14.3 Unusual muscle involvement observed in FSHD patients

14.3.1 Facial-sparing scapular myopathy

Scapular winging due to involvement of scapula fixators is a hallmark feature of FSHD, but may also be a prominent finding in other muscular disorders including Emery–Dreyfuss muscular dystrophy, congenital myopathies, myotonic dystrophy and acid maltase deficiency (Barohn *et al.*, 1993; Kissel, 1999). In the absence of facial muscle involvement, a diagnosis of FSHD would be difficult.

Jardine *et al.* (1994b) described a 4q-linked family including seven affected individuals in two generations. The patients showed scapular onset muscular dystrophy without facial involvement. Weakness began in the shoulders between 12 and 40 years of age. There was no distal weakness in the upper or lower extremities and there were no sensory abnormalities. In several cases, there was marked

asymmetry with weakness on the right side more than on the left. There was no demonstrable facial weakness in any of the affected individuals.

Felice *et al.* (2000) performed genetic analysis on 14 patients with facial-sparing scapular myopathy, and determined that 71% of them had a short *Eco*RI fragment of less than 40 kb. These patients were estimated to constitute approximately 15% of FHSD patients. The clinical symptoms of the patients other than facial muscle involvement resembled typical FSHD patients in age at onset, physical characteristics, and association between fragment size and disease severity.

14.3.2 Tongue atrophy

Although involvement of facial muscles occurs in the majority of patients with FSHD, weakness of extraocular, masticatory, pharyngeal and lingual muscles are considered to be the exclusion criteria of the disease. However, some reports described the involvement of the tongue. Shimizu *et al.* (1991) reported a patient with 'congenital FSHD' with tongue atrophy. His father showed similar but milder muscle atrophy of the face and shoulder girdle since adolescence. The patient presented facial muscle weakness since birth, and then developed wasting around the neck, shoulder girdle, upper arms and thighs. Calf hypertrophy was also observed. Hearing disturbance was detected at the age of 6 years, and he also noted atrophy of the tongue and the bilateral thighs at the age of 10. EMG in the extremities and the tongue revealed myopathic changes. Goto and Sugihara (1994) also reported a patient diagnosed as congenital FSHD. Her mother had moderate facial weakness and mild proximal weakness of the upper and lower limbs. The patient presented bilateral facial weakness, tongue atrophy and weakness of the shoulder girdle, upper arms, and thighs, and bilateral mild sensorineural hearing loss.

Yamanaka *et al.* (2001) observed that 4.6% (7/151) of Japanese patients with FSHMD1A had tongue atrophy with abnormal MRI findings and typical myogenic patterns of electromyography (*Figure 14.1*). All seven patients belong to a group of early-onset FSHD and the *Eco*RI fragment size varied from 10 to 17 kb. They suggested that the FSHD patients, especially with a large gene deletion on chromosome 4q35 could have myopathic tongue atrophy.

14.3.3 Head drooping

Ichikawa *et al.* (1996) described three unrelated patients with FSHD showing conspicuous head drooping caused by severe wasting of posterior neck muscles. These patients realized abnormal neck posture much earlier than appearance of obvious gait disability, while they show other characteristic FSHD features. Other affected members from the same families did not show abnormal head drooping.

14.3.4 Abdominal muscle involvement and lumbar lordosis

Awerbuch *et al.* (1990) reported that the Beevor sign is commonly observed in patients with FSHD but not in other types of neuromuscular disorder. This sign was originally proposed by English neurologist C.E. Beevor as an indication of the level of involvement in spinal cord lesions. The umbilicus moves upward when the subject in the supine position raises their head because of weakness of the lower

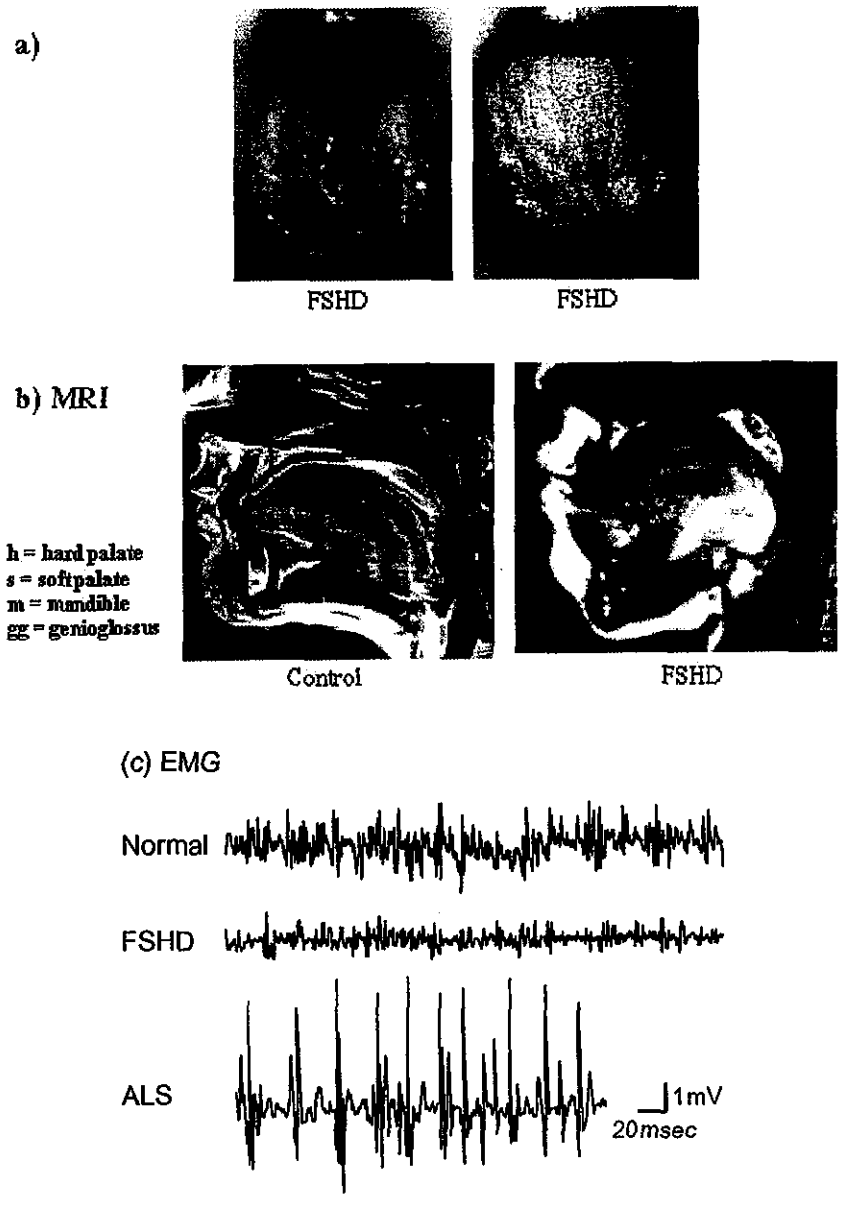

Figure 14.1 (A) Tongue pictures of FSHD patients with tongue atrophy (Yamanaka *et al.*, 2001). (B) Tongue MRI of a normal control and an FSHD patient. The normal tongue virtually fills the entire oral cavity. Its internal structure shows two curvilinear bands (arrows) parallel to the mucosal surface. In FHSD, there are scattered abnormal high-intensity areas (asterisks) in the internal tongue structure. The two curvilinear bands (arrows) are deranged and show disorganization of the tongue architecture. The atrophic tongue produces a space in the upper oral cavity (Yamanaka *et al.*, 2001). (C) In the FSHD patient, EMG showed typical myogenic changes, whereas a patient with amyotrophic lateral sclerosis (ALS) showed neurogenic changes.

rectus abdominis muscles. In FSHD patients, this sign appears even before functional weakness of abdominal wall muscles is apparent.

Early involvement of the abdominal muscles with relative sparing of the psoas major muscle in FSHD patients was detected by CT scan (Horikawa *et al.*, 1992). This may exacerbate lumbar lordosis, the most common form of spinal deformity in the patients (Kilmer *et al.*, 1995).

14.3.5 Limb girdle type muscle weakness

Limb girdle muscular dystrophy (LGMD) is a group of genetically heterogeneous progressive muscular disorders predominantly involved in proximal limb muscles. The genes responsible and their protein products have been identified in at least three autosomal dominant and ten autosomal recessive forms.

Some 4q35-linked FSHD patients were reported to display limb girdle type muscular weakness. The initial symptoms of the patients were weakness of the proximal lower limb muscles, and complaint of difficulty in climbing stairs and walking. Facial muscle involvement was absent or very mild (Nakagawa *et al.*, 1996, 1997; van der Kooi *et al.*, 2000; Felice and Moore, 2001). Although detailed genetic analysis was not performed, Kazakov and Rudenko (1995) also reported a clinically and genetically homogeneous group of patients with autosomal-dominant inheritance manifesting a gradually descending form of FSHD, called facioscapulolimb dystrophy (FSLD).

Reardon *et al.* (1991) reported a 32-year-old male patient who had typical calf hypertrophy and limb girdle type of muscle weakness. These findings suggested Becker muscular dystrophy (BMD), although he presented with sudden onset of facial weakness at age of six. When his daughter showed facial weakness, autosomal dominant FSHD became most likely. Since calf hypertrophy, although rare, has been reported in FSHD, differentiation between FSHD and BMD may also be difficult in an isolated male patient.

14.3.6 Distal myopathy

Some patients with FSHD presented foot drop by virtue of weakness of the foot extensor muscle. Padberg investigated 107 patients and found foot extensor weakness in 8% (Padberg, 1982). Felice and Moore (2001) reported a 78-year-old woman who was followed for 15 years with a diagnosis of late-onset autosomal dominant distal myopathy. The patient showed progressive bilateral foot drop, and later developed difficulties climbing stairs. Although mild eye-closure weakness and late-onset sensorineural hearing loss were observed, the patient showed no other clinical characteristic features of FSHD. Her mother had similar problems with mild facial muscle involvement and hearing loss. The patient was believed to have a form of hereditary distal myopathy, although genetic analysis revealed this proband to have a 30 kb *Eco*RI fragment.

Involvement of calf muscles is presumed to be affected only in later stages of the disease as compared with the anterior tibial muscle. CT scans also revealed a relatively mild involvement of the gastrocnemius and soleus muscles as compared with the tibialis anterior muscle (Horikawa *et al.*, 1992). However, van der Kooi *et al.* (2000) reported a male patient who initially experienced foot pain and inability to

walk on his toes at the age of 50 owing to calf muscle involvement. The *Eco*RI fragment size of this patient was 20 kb.

14.4 Muscle pain

Muscle pain is rarely described as a symptom of FSHD. Some reports described the association of muscle pain and weakness of facioscapulohumeral distribution, but they were unable to distinguish between FSHD and polymyositis (Rothstein *et al.*, 1971; Munsat *et al.*, 1972; Bates *et al.*, 1973; Bacq *et al.*, 1985). van der Kooi *et al.* (2000) reported a patient showing mild shoulder symptoms such as tiredness and pain. The *Eco*RI fragment size exhibited by this patient was 38 kb (van der Kooi *et al.*, 2000). These authors also described another patient with a 20 kb *Eco*RI fragment who initially exhibited foot pain and calf muscle weakness.

Bushby *et al.* (1998) reported four adult patients with FSHD showing muscle pain, which remains their most disabling symptom. Three of them had a short *Eco*RI fragment from 20 to 24 kb, but one manifested only normal-sized fragments. All patients reported between three and seven different pains of varying site and nature, and none had more than one painfree day per month and all complained of disturbed sleep. These myalgic pains could be particularly difficult to control by analgesic or anti-inflammatory therapy. They concluded that muscle pain in FSHD is an under-reported but significant symptom.

14.5 Association with other types of neuromuscular disorders

Tonini *et al.* (2002) reported two unique Brazilian families with FSHMD1A and other forms of muscular dystrophy in the same family. In the first, the 35-year-old male proband had limb girdle muscular dystrophy with proximal weakness, elevated creatine kinase and a myopathic muscle biopsy. All the proteins known to be associated with limb girdle muscular dystrophy were normal. Two of his sisters also complained of muscle weakness. The oldest sister exhibited clinical signs consistent with FSHD, and had a 30 kb *Eco*RI/*Bln*I fragment which was found in another six relatives, but surprisingly not in the affected proband or the other sister. In the second family, a 57-year-old male with a typical FSHD phenotype had a 17 kb *Eco*RI/*Bln*I fragment which was also present in other affected relatives. However, in a 14-year-old severely affected male cousin, confined to a wheelchair since age 12, but without facial weakness, the small fragment was absent. In case of these rare associations, it may be important to perform genetic tests in all affected individuals in a family.

Sakuma *et al.* (2001) reported a male patient with FSHD accompanied by myasthenia gravis. The patient had a 35 year history of FSHD and his mother was also affected. At the age of 50, the proband was admitted to hospital because of acute progression of muscle weakness without any fluctuation. No blepharoptosis or ocular movement disturbance was observed. However, disturbance in chewing and swallowing appeared about a month after admission, an uncommon finding in FSHD. The diagnosis of myasthenia gravis was confirmed by the repetitive stimulation test, edrophonium chloride injection, and by titre of serum anti-Ach receptor antibody (Sakuma *et al.*, 2001).

14.6 Cardiac involvement

Involvement of cardiac muscle and serious electrocardiographic abnormalities are rare in FSHD, and are not considered to be part of the disease (de Visser *et al.*, 1992; Kilmer *et al.*, 1995). However, some reports have described FSHD patients with serious arrhythmia. These patients required pacemaker implantation because of symptomatic atrial tachycardia or complete A–V block (Ohno *et al.*, 1991; Shen and Madsen, 1991).

Also, a unique family with both FSHMD1A and hereditary long QT syndrome (LQT) was reported. In this family, five individuals in three generations were diagnosed as having FSHMD1A by clinical and genetic analysis, and three of the five affected members were also diagnosed as having LQT. LQT constitutes a group of disorders that cause syncope and sudden death from ventricular arrhythmia in an autosomal dominant fashion. One of the loci for LQT (LQT4) was mapped to chromosome 4q25–q27, and possible linkage between FSHD and LQT was speculated upon (Kimura *et al.*, 1997). Recently, an ankyrin B gene mutation was identified in the large French family with LQT4 (MIM:600919) (Mohler *et al.*, 2003). Genetic analysis should clarify the association of FSHD and LQT4 in this family.

Possible cardiomyogenic involvement in FSHD was also reported using Thallium-201 single-photon-emission computed tomography (Tl-201-SPECT). Yamamoto *et al.* (1986) reported abnormal reduced Tl-201 uptake in 71% of FSHD patients that were scattered in all left ventricular wall segments. Faustmann *et al.* (1996) revealed stress-induced reduced Tl-201 uptake in the affected members of a 4q35-linked FSHD family and concluded that careful supervision of cardiac functions may be needed for FSHD patients.

Further, the thoracic deformity observed in FSHD may cause cardiac problems. Nakayama *et al.* (1999) studied electrocardiogram (ECG) and ECG-gated cardiac magnetic resonance imaging (MRI) in eight patients with FSHD. The patients frequently showed ECG abnormalities including elevated P wave and multifocal atrial premature contractions, together with restricted right ventricular movement and enlarged right atrium. Similar changes are often observed in patients suffering from severe 'funnel chest' or 'straight back syndrome'. The authors concluded that the characteristic thoracic deformity may play a primary role in the development of cardiac problems in FSHD (Nakayama *et al.*, 1999).

14.7 Respiratory failure

Pulmonary dysfunction in FSHD is usually mild, if present. However, some patients show progressive, life-threatening respiratory failure. Yasukohchi *et al.* (1988) described two sibs with FSHD. The 8-year-old sister had only muscle manifestations, whilst the brother, aged 13 years, manifested sensorineural hearing loss and marked tortuosity of retinal arterioles. He also showed early onset and progression of severe restrictive pulmonary dysfunction, and cor pulmonale, which led to death. Nakagawa *et al.* (1996) also reported an early-onset FSHD patient with respiratory failure and retinal vasculopathy. Interestingly, the 53-year-old mother

of the proband with the same genetic abnormality had limb girdle type muscular weakness with very mild facial involvement.

Kilmer *et al.* (1995) reported that nearly 50% of the FSHD patients had vital capacity evidence of restrictive lung disease. However, only 13% had severe involvement, and only 22% had a history of pulmonary complications. There was no age or disease duration effect on pulmonary function measurements or complications. They concluded that maximal expiratory pressure measurements on the FSHD patients were more sensitive than other pulmonary function tests, as with the other neuromuscular diseases (Kilmer *et al.*, 1995).

14.8 Central nervous system involvement

FSHD patients usually exhibit little or no cognitive impairment (Sigford and Lanham, 1998). However, the patients with a large deletion in the FSHD gene region tend to have a higher chance of showing severe clinical phenotypes with central nervous system abnormalities. Akiyama *et al.* (1991) reported a female patient with FSHD with sensorineural deafness, retinal vessel abnormality, mental retardation and epilepsy with infantile spasms at 6 months of age.

Miura *et al.* (1998) reported two unrelated, severely affected patients with mental retardation and epilepsy. One patient showed infantile spasms at the age of 4 months and localization-related epilepsy at the age of 2.5 years. Muscular atrophy in the face, shoulder girdle and upper arms was observed from the age of 4 years. In the other patient, lack of facial expression was noticed from the age of 1 year, and at 4 years she was noted to have a loss of bilateral upward gaze. She developed localization-related epilepsy at the age of 9 years. From the age of 10 years, weakness of the lower limbs progressed and she became wheelchair-bound at the age of 14 years and 8 months. She had moderate sensorineural hearing loss, a loss of bilateral upward gaze and tongue atrophy. Their IQs were 33 and 45, respectively. Southern blot analysis revealed a 10 kb *Eco*RI fragment in both patients (Miura *et al.*, 1998).

Funakoshi *et al.* (1998) found nine patients with the smallest *Eco*RI fragments (10–11 kb), and reported that all of them were classified as having the early-onset form. These patients exhibited a high frequency of both epilepsy (4/9, 44%) and mental retardation (8/9, 89%). These two reports concluded that mental retardation and epilepsy may be part of the clinical spectrum of FSHD, especially in the early-onset form with a large deletion.

14.9 Psychopathological and emotional examination

Bungener *et al.* (1998) performed psychological studies on patients with 11 FSHD, together with 15 myotonic dystrophy and 14 healthy subjects. A semistructured interview was used to determine DSM III-R criteria for major depressive episodes, dysthymic episodes, and generalized anxiety. The Montgomery and Asberg, and the Hamilton depressive scales, the Covi and Tyrer anxiety scales, the Abrams and Taylor scale for emotional blunting, and the depressive mood scale were all used in the study. The results indicated that the patients with FSHD were the most depressed and most anxious.

14.10 Hearing loss

Meyerson *et al.* (1984) reported sensorineural hearing loss in two sibs with FSHD. Gieron *et al.* (1985) described a mother and three children with FSHD, sensorineural hearing loss, and marked tortuosity of retinal vessels. The deafness, which varied from mild to moderate, was bilateral and early in onset; audiological studies indicated the cochlea to be the site of the abnormality. Matsuzaka *et al.* (1986) reported a sporadic patient with early-onset FHSD, sensorineural hearing loss, mental retardation, and marked tortuosity of the retinal arterioles. Fujimura *et al.* (1989) also reported a sporadic case of a 12-year-old boy with FSHD, sensorineural hearing loss and exudative angioma of bilateral retina. His hearing loss was noted at 9 years, followed by muscle weakness of his right upper extremity at 11 years.

Voit *et al.* (1986) found bilateral sloping high frequency hearing loss of 20–90 dB in 6/10 patients with infantile- or adolescent-onset FSHD. In some patients, the hearing loss was clearly progressive. The outer hair cells of the basal turn were predominantly affected. The authors concluded that cochlear dysfunction is a specific and frequent phenomenon of early-onset FSHD.

Brouwer *et al.* (1991) performed screening audiometry in 56 patients with autosomal dominant FSHD and suggested that the change of hearing function between 4000 Hz and 6000 Hz is part of the disease and may lead to severe hearing loss in some patients. Generally, the patients show bilateral high-tone hearing loss, but some showed also at the lower (speech) frequencies.

Brain stem auditory-evoked potentials were generally normal (Verhagen *et al.*, 1995), but some patients exhibited abnormal increased threshold and prolonged latency (Takeya *et al.*, 1990; Fierro *et al.*, 1997).

The frequency of hearing loss was estimated to be about 25–64% of affected patients, and is now considered to be an important feature of FSHD (Sanchez-Alcon *et al.*, 1994; Padberg *et al.*, 1995a). Age and severity of the myopathy did not have a clear relationship with the hearing loss (Sanchez-Alcon *et al.*, 1994; Padberg *et al.*, 1995a). On the other hand, Rogers *et al.* (2002) undertook detailed pure tone audiometric examination in 21 adult-onset FSHD cases and found no significant difference in the prevalence of hearing impairment. They concluded that hearing impairment is not common in adult-onset facioscapulohumeral muscular dystrophy. Moderate to severe sensorineural deafness is, however, common in early-onset FSHD.

14.11 Retinopathy

Retinal vasculopathy is known to be associated with FSHD. Association of FSHD and Coats' syndrome (exudative retinopathy with telangiectasis, sometimes causing blindness) was reported especially in the severe early-onset form with mental retardation (Small, 1968; Taylor *et al.*, 1982; Voit *et al.*, 1986).

Gurwin *et al.* (1985) reported a 22-year-old FSHD patient with a macular lesion in her right eye and poor central vision, which had been present since early childhood. Fluorescein angiographic examination revealed bilateral peripheral vessel

closure, peripheral retinal telangiectasis, and hyperfluorescence in both foveae. Three affected family members also had clinical deafness and abnormal retinal vasculature, as determined by fluorescein angiography, but none had related visual symptoms. The authors concluded that in young patients with unexplained retinal vascular lesions, the diagnosis of FSHD should be considered (Gurwin *et al.*, 1985).

Fitzsimons *et al.* (1987) found peripheral retinal capillary abnormalities including telangiectasia, closure, leakage and microaneurysm formation in 56 of 75 individuals with clinical or genetic evidence of FSHD. Retinal vasculopathy may present early in life and before there is overt evidence of muscle disease. However, there was no correlation between the severity of the muscle disease and the extent of the retinal vascular abnormality (Fitzsimons *et al.*, 1987). Padberg *et al.* (1995b) also reported similar retinal vasculopathy including telangiectasia and microaneurysms in 49% of patients with FSHD by using fluorescein retinal angiography.

The risk to vision has not been established since there are only few reports of severe visual loss in FSHD. Pauleikhoff *et al.* (1992) reported two cases of young girls who developed FSHD and exudative retinal detachment due to telangiectasis. In the first patient, the severity of the disease precluded visual recovery despite extensive photo- and cryotherapy. In the other, visual acuity in both affected eyes was retained after treatment (Pauleikhoff *et al.*, 1992).

Since visual loss may be preventable, ophthalmic examination should be undertaken on infants and young children at risk of having a deletion of the FSHD region, although visual complications of telangiectasis are rare (Fitzsimons *et al.*, 1987; Pauleikhoff *et al.*, 1992).

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Reduction of insulin-stimulated glucose uptake by peroxynitrite is concurrent with tyrosine nitration of insulin receptor substrate-1☆

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Abstract

Inducible nitric oxide synthetase plays an essential role in insulin resistance induced by a high-fat diet. The reaction of nitric oxide with superoxide leads to the formation of peroxynitrite ($ONOO^-$), which can modify several proteins. In this study, we investigated whether peroxynitrite impairs insulin-signalling pathway. Our experiments showed that 3-(4-morpholinyl)sydnonimine hydrochloride (SIN-1), a constitutive producer of peroxynitrite, dose-dependently inhibited insulin-stimulated glucose uptake. While SIN-1 did not affect the insulin receptor protein level and tyrosine phosphorylation, it reduced the insulin receptor substrate-1 (IRS-1) protein level, and IRS-1 associated phosphatidylinositol-3 kinase (PI-3 kinase) activity. Although SIN-1 did not induce Ser^{307} phosphorylation of IRS-1, tyrosine nitration of IRS-1 was detected in SIN-1-treated-Rat1 fibroblasts expressing human insulin receptors. Mass spectrometry showed that peroxynitrite induced at least four nitrated tyrosine residues in rat IRS-1, including Tyr^{939}, which is critical for association of IRS-1 with the p85 subunit of PI-3 kinase. Our results suggest that peroxynitrite reduces the IRS-1 protein level and decreases phosphorylation of IRS-1 concurrent with nitration of its tyrosine residues.

Keywords: Insulin resistance; Oxidative stress; Insulin receptor substrate-1; Nitric oxide; iNOS; Peroxynitrite

Nitric oxide (NO) has many physiological functions in the central nervous system, cardiovascular system, and immune system, acting as a signal transduction molecule to control diverse biological functions such as vasodilatation [1] and the secretion of neurotransmitters [2]. On the other hand, excessive NO production is involved in inflammation, and thus plays an important role in the pathophysiology of various diseases. Among the three isoforms of nitric oxide synthetase (NOS), inducible NOS (iNOS) is known to produce far larger amounts of NO compared with the other isoforms, and iNOS has been recognized to play a role in the pathogenesis of inflammatory and autoimmune diseases, such as septic shock, hemorrhagic shock, rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, and

☆ *Abbreviations:* BSA, bovine serum albumin; CBB, Coomassie brilliant blue R250; Da, Dalton; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Erk, extracellular signalling-regulated kinase; FCS, fetal calf serum; HIRc, rat-1 fibroblasts expressing human insulin receptors; iNOS, inducible NOS; IRS, insulin receptor substrate; LC, liquid chromatography; MAPK, mitogen-activated protein kinase; MS, mass spectrometry; NO, nitric oxide; NONOate, (*Z*)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino}-diazen-1-ium-1,2-diolate; NOS, nitric oxide synthetase; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PDA, polydiacetylene; PI3-kinase, phosphatidylinositol-3 kinase; PMSF, phenylmethylsulphonyl fluoride; SIN-1, 3-(4-morpholinyl)sydnonimine hydrochloride; TFA, trifluoroacetic acid; TNF, tumour necrosis factor.

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multiple sclerosis [3,4]. NO can also be viewed as a radical containing an unpaired electron. In addition to reacting with superoxide (O_2^-), NO is transformed into peroxynitrite ($ONOO^-$) [5], which is a highly reactive radical.

Peroxynitrite can modify tyrosine residues and form nitrotyrosine in various proteins. Nitration of protein tyrosine residues can lead to damage that alters protein function and stability [6]. It also often affects signal transduction pathways by disrupting tyrosine phosphorylation [7,8], because tyrosine phosphorylation is commonly involved in signal transduction. For example, the insulin signal transduction pathway involves the tyrosine phosphorylation of several proteins, so peroxynitrite might impair the action of insulin through nitration of key tyrosine residues on the proteins involved in insulin signalling.

Tumour necrosis factor-α (TNF-α) causes insulin resistance in obese subjects and probably promotes the expression of iNOS [9]. Recently, Perreault and Marette [10] found that targeted disruption of *iNOS* was almost completely protective against high-fat diet-induced insulin resistance in mice. This finding suggests that activation of iNOS per se plays a key role in high-fat diet-induced insulin resistance in vivo although its mechanism has not yet been fully elucidated.

The present study was designed to investigate whether peroxynitrite induced insulin resistance. Our results indicated that peroxynitrite could impair insulin-stimulated glucose uptake by 3T3-L1 adipocytes. Analysis of the insulin signal transduction pathway revealed marked repression of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol-3 (PI3)-kinase activity after exposure to peroxynitrite. These changes were concurrent with a reduction of the IRS-1 protein level and tyrosine phosphorylation of this protein. In addition, mass spectrometric analysis (MS) revealed that exposure of rat IRS-1 to peroxynitrite provoked the nitration of at least four tyrosine residues including Tyr^{939}, which is one of the critical docking sites of the p85 subunit of PI3-kinase.

Materials and methods

Antibodies and reagents. Human insulin was purchased from Wako Chemicals (Tokyo, Japan). Anti-nitrotyrosine antibody, anti-IRS-1 antibody, anti-p85 antibody, anti-serine (307)-phosphorylated IRS-1 antibody, and rat recombinant IRS-1 were obtained from Upstate Biotechnology Incorporated (Lake Placid, NY). Anti-phosphotyrosine (PY69), anti-insulin receptor β subunit antibody, and anti-Akt1/2 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-threonine (308), serine (473) phosphospecific Akt antibody, and anti-phosphospecific extracellular signalling-regulated kinase (Erk) antibody were from Cell Signaling Technology (Beverly, MA). TNF-α was purchased from R&D Systems (Minneapolis, MN). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were purchased from Bio-Rad Laboratories (Tokyo). 3-(4-Morpholinyl)sydnonimine hydrochloride (SIN-1) and peroxynitrite were obtained from Dojin Tech. (Kumamoto, Japan). (*Z*)-1-{*N*-[3-Aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino}-diazen-1-ium-1, 2-diolate (spermine NONOate) was purchased from Alexis Biochemicals (Tokyo).

Cell culture. Preadipocyte-derived 3T3-L1 cells (purchased from ATCC) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). To induce differentiation into adipocytes, the cells were incubated with isobutylmethylxanthine (500 μM), dexamethazone (25 μM), and insulin (4 μg/ml) for 3 days and then with insulin alone for another 3 days until more than 95% cells had differentiated. Then the cells were trypsinized and reseeded in appropriate culture dishes for further experiments [11].

Rat1 fibroblasts expressing human insulin receptors (HIRc) provided by Dr. J.M. Olefsky were grown and maintained in low-glucose DMEM (Invitrogen, San Diego, CA) containing 50 U/ml streptomycin and 10% FCS under a 10% CO_2 environment.

2-Deoxyglucose uptake. Glucose uptake was measured by the methods of Klip et al. [12] with minor modifications. 3T3-L1 adipocytes were incubated with or without SIN-1, spermine NONOate or MnTBAP for 12 h in high-glucose DMEM with 2% FCS, followed by incubation in glucose-free DMEM supplemented with 0.2% bovine serum albumin (BSA) in the absence or presence of 100 nM insulin for 1 h at 37 °C. After the addition of 10 μl of 2-[^{3}H]deoxyglucose or L-[^{3}H]deoxyglucose (0.1 μCi), the cells were incubated for 5 min and glucose uptake was determined from the 2-[^{3}H]deoxyglucose counts in each cell lysate. To obtain a concentration at which cell membrane transport was rate-limited, the value for L-glucose was subtracted to correct each sample for the contributions of diffusion and trapping. The data were corrected by the protein concentrations measured by the method of Bradford, as described previously [13].

Immunoprecipitation assay and Western blotting. 3T3-L1 adipocytes and HIRc cells were serum-starved for 12 h in a medium with or without 4 or 8 mM SIN-1. Then the cells were stimulated with 100 nM insulin for 5 or 20 min at 37 °C. After washing three times with ice-cold phosphate-buffered saline (PBS), the cells were lysed in lysis buffer (20 mM Tris–HCl [pH 7.5], 1 mM ethylenediaminetetraacetic acid [EDTA], 140 mM NaCl, 1% Nonidet P-40 [NP-40], 1 mM Na_3VO_4, 1 mM phenylmethylsulphonyl fluoride [PMSF], 50 mM NaF, and 10 μl/ml proteinase cocktail [Sigma, St. Louis, MO]). Cellular lysates were obtained after sonication and centrifugation. For the immunoprecipitation assay, lysates containing 1 mg of total protein were incubated with the indicated antibodies for 12 h at 4 °C. Especially, to elucidate the effect of SIN-1 on insulin signalling directly associated with IRS-1, we used half dose of anti-IRS-1 antibody as recommended by the manufacturer to obtain a similar amount of IRS-1 protein from each sample. After collection on protein A or G Sepharose, the immune complexes were washed three times with 1 ml lysis buffer, and Western blotting analysis was performed as described previously [13].

PI3-kinase assay. 3T3-L1 adipocytes were serum-starved for 16 h in the presence or absence of 4 mM SIN-1. Then the cells were incubated in the presence or absence of 100 nM insulin for 20 min. After washing twice with ice-cold washing buffer (20 mM Tris–HCl [pH 7.5], 1 mM $MgCl_2$, 1 mM $CaCl_2$, 137 mM NaCl, and 100 μM Na_3VO_4), the cells were lysed with lysis buffer (washing buffer plus 10% glycerol, 1% NP-40, 1 mM PMSF, and 10 μl/ml proteinase cocktail). After sonication and centrifugation, the supernatant (containing 1.5 mg protein) was incubated with anti-IRS-1 antibody for 2 h and then incubated with protein A–Sepharose for 12 h at 4 °C. The resulting immunoprecipitates were washed three times with PBS containing 1% NP-40, 100 μM Na_3VO_4, three times with 100 mM Tris–HCl (pH 7.5), 500 mM LiCl, and 100 μM Na_3VO_4, and twice with 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 100 μM Na_3VO_4. Pellets were suspended in 50 μl of 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 100 μM Na_3VO_4. The reaction was initiated by the addition of 200 μM ATP, 1.11 MBq [γ-^{32}P]ATP, 10 mM $MgCl_2$, and 10 μg

phosphatidylinositol. Incubation was performed at 30 °C for 10 min and the reaction was terminated with the addition of 8 N HCl. After extraction with $CHCl_3$ and CH_3OH (1:1), the aqueous phase was applied to a silica gel thin-layer chromatography plate. The plate was developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (100:70:15:25), dried, visualized, and quantified with a BAS2500 (Fuji film, Tokyo).

Separation of recombinant IRS-1 by 1-dimensional SDS–polyacrylamide gel electrophoresis. The gel for separation of IRS-1 was prepared as described previously [14]. The electrophoresis apparatus was a product of Nihon Eido (Tokyo) and a gel size measuring 130 × 130 × 1 mm was used for 1-dimensional SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (12 lanes). The concentrations of polyacrylamide in the separating and stacking gels were 6% and 3%, respectively, while that of polydiacetylene (PDA) as a cross-linker was 2.6%/acrylamide. One milligram of rat recombinant IRS-1 (Upstate Biotechnology) was incubated in PBS with or without 50 μM peroxynitrite for 1 h at 4 °C. For immunoprecipitation, the samples were incubated with 4 μg anti-IRS-1 antibody and collected on protein A–Sepharose. For in-gel digestion, immunoprecipitates were washed three times with PBS and boiled with modified Laemmli sample buffer (62.5 mM Tris–HCl [pH 8.5], containing 10% glycerol, 2% SDS, 0.1 M dithiothreitol [DTT], and 0.0025% bromophenol blue). After cooling at room temperature, 5 μl aliquots of 30% acrylamide stock solution were added for in situ alkylation of the cysteinyl residues of proteins. Immediately after mixing, the samples were loaded onto a stacking gel. SDS–PAGE was commenced at 5 mA for 1 h in order to concentrate the proteins in the stacking gel and then the proteins were separated at 10 mA per gel for 2 h. The running buffer consisted of 25 mM Tris and 19.2 mM glycine (pH 8.45) containing 0.1% SDS, according to Laemmli [15].

In-gel trypsin digestion of IRS-1. After completing SDS–PAGE, proteins were visualized by staining with Coomassie brilliant blue R250 (CBB). The approximately 150 kilodalton (kDa) band was excised with a razor blade and cut into small pieces, which were placed in an Eppendorf tube, and washed with 50% acetonitrile and 100 mM ammonium bicarbonate in purified water for 10 min at 37 °C. Then the gel pieces were dehydrated by addition of acetonitrile for 10 min at 37 °C and dried in a vacuum centrifuge (Micro Vac MV-100; Tomy, Tokyo). The dried samples were immersed in 30–50 μl trypsin at 10 ng/μl, dissolved in 100 mM ammonium bicarbonate (pH 8.5), and let to stand for 15–18 h at 37 °C. The trypsinized peptides were extracted with the following solutions (50 μl of each) for 10 min at 37 °C: (i) 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA), (ii) a mixture of isopropanol:formic acid:acetonitrile:purified water (15:20:25:40 v/v), and (iii) 80% acetonitrile. Extracts were evaporated to dryness in a vacuum centrifuge and used to identify IRS-1 by MS.

Identification of IRS-1 and its nitrated tyrosine residues by MS. Peptide mapping was carried out using the APIQSTAR Pulsar hybrid mass spectrometer system (Foster City, CA) with a micro liquid chromatograph (LC) (Magic 2002; Michrom BioResource, Auburn, CA). The conditions for micro LC were as follows: a Magic C18 column (0.2 mm ID × 50 mm) was eluted with 0.1% formic acid in solvent A, and 0.1% formic acid in 90% acetonitrile (solvent B) using the following program; 5% solvent B for 5 min, gradient at 2.1%/min for 45 min, 100% solvent B for 5 min at a flow rate of 2.5 μl/min. The MS conditions were as follows: ion spray voltage 3.0 kV, electron multiplier voltage 2400 V, curtain gas nitrogen 10 for MS and MS/MS analysis; collision gas, nitrogen 10, and collision energy, 20–25 eV for MS/MS analysis. To identify the proteins, peptide mapping of the 150 kDa band was performed by LC-MS using the PROWL (ProFound) search engine (prowl.rockefeller.edu/cgi-bin/ProFound) and the public domain database (NCBI) available on the internet. The major ion peaks of the total ion chromatogram were further analysed to obtain the amino acid sequences of the trypsinized peptides by LC-MS/MS using the Mascot search engine (www.matrixscience.com) under the same conditions (QSTAR with Magic micro LC) and the same database.

The nitrated tyrosine residues in the peptides were identified by single-ion monitoring (SIM) using $[M+2H]^{2+}$ and $[M+45(NO_2)+2H]^{2+}$ ions. The nitrated tyrosine peptides appeared on the chromatogram at 30–150 s after the unmodified tyrosine peptides [16].

Statistical analysis. All results are expressed as means ± SEM. Differences between groups were examined for significance using Student's *t* test and a *P* value of less than 0.05 was taken to indicate the presence of a significant difference.

Results

Peroxynitrite impairs insulin-stimulated glucose uptake

To investigate the effects of peroxynitrite on insulin-stimulated glucose uptake, we incubated 3T3-L1 adipocytes with various concentrations of SIN-1 for 12 h (Fig. 1) and then measured glucose uptake using 2-deoxyglucose as a tracer. SIN-1 simultaneously generates superoxide and NO, thus peroxynitrite is produced by

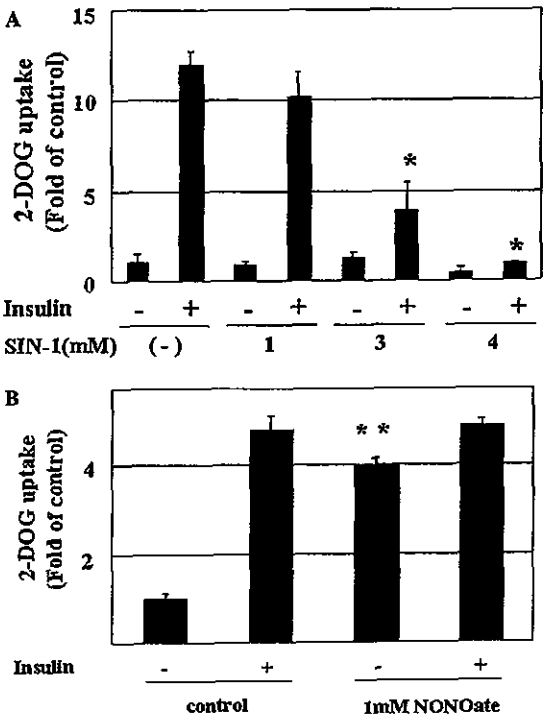

Fig. 1. Effects of peroxynitrite on insulin-stimulated 2-deoxyglucose uptake. (A) 3T3-L1 adipocytes were incubated with or without the indicated concentrations of SIN-1 for 12 h. (B) 3T3-L1 adipocytes were incubated with or without 1 mM spermine NONOate for 12 h. Then cells were stimulated with 100 nM insulin for 1 h after which 2-deoxyglucose uptake was measured as described in Materials and methods. The results were corrected by the protein concentration. 2-Deoxyglucose uptake was expressed relative to that by cells incubated in the absence of insulin and SIN-1. All experiments were performed in duplicate on five separate occasions. Data are means ± SEM. Statistical significance was determined by Student's *t* test. $^*p < 0.05$ compared with insulin-stimulated control cells. $^{**}p < 0.05$ compared with insulin-unstimulated control cells.

rapid chemical reaction in treated cells. In unstimulated adipocytes, treatment with SIN-1 did not cause a significant change of glucose uptake. On the other hand, treatment with SIN-1 induced significant dose-dependent inhibition of insulin-stimulated glucose uptake (control, 11.93 ± 0.78; SIN-1 at 1 mM, 10.20 ± 1.27; SIN-1 at 3 mM, 3.84 ± 1.58; and SIN-1 at 4 mM, 0.92 ± 0.06. Note: values are expressed relative to the 2-deoxyglucose uptake by cells without insulin or SIN-1 treatment, Fig. 1A).

Next, to confirm that the effect of SIN-1 on insulin signalling is caused by peroxynitrite rather than by the intermediate product, NO, we treated differentiated 3T3-L1 adipocytes with 1 mM spermine NONOate which is a constitutive NO producer, for 12 h. NO stimulated glucose uptake without insulin (Fig. 1B). Glucose uptake stimulated by insulin was not different in adipocytes treated with and without NONOate (Fig. 1B) as described previously [17]. These data indicate that peroxynitrite not NO impairs insulin-stimulated glucose uptake.

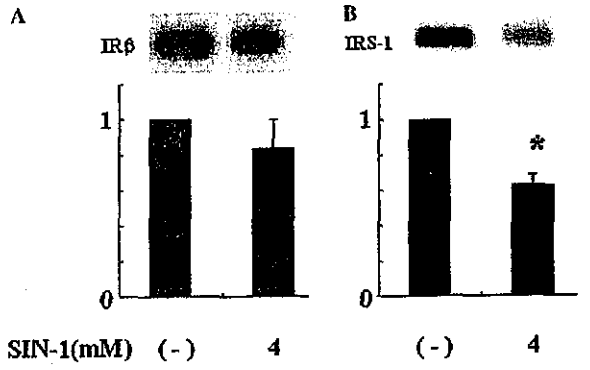

Fig. 2. Effects of peroxynitrite on insulin receptor and IRS-1 protein levels. 3T3-L1 adipocytes were serum-starved and incubated with or without 4 mM SIN-1 for 12 h. Cell extracts were immunoblotted with anti-insulin receptor antibody (A) or anti-IRS-1 antibody (B). Results are from three independent experiments. The intensity of each band was quantitated relative to the band for cells incubated in the absence of SIN-1. Data are means ± SEM. Statistical significance was determined by Student's t test. *$p < 0.05$ compared with insulin-stimulated control cells.

Peroxynitrite impairs IRS-1-associated PI3-kinase activity

The next series of experiments were designed to determine the target molecule for peroxynitrite-induced changes of glucose uptake. For this purpose, 3T3-L1 adipocytes were cultured with 4 mM SIN-1 and SIN-1-induced changes of the insulin-signalling pathway were investigated. SIN-1 decreased the IRS-1 protein level by 38%, but not the insulin receptor level (Fig. 2). Thus, the decrease of insulin-stimulated glucose uptake by SIN-1 treatment seemed to be at least partly caused by the decrease of IRS-1 protein. To investigate other modifications of the insulin signal transduction pathway by peroxynitrite, we measured insulin-stimulated tyrosine phosphorylation using a similar amount of immunoprecipitated IRβ or IRS-1 protein to SIN-1-untreated cells. Peroxynitrite did not cause a significant change of insulin-stimulated tyrosine phosphorylation of the insulin receptor (Fig. 3A). On the other hand, SIN-1 suppressed insulin-induced stimulation of tyrosine phosphorylation of IRS-1 by 43% (control, 5.57 ± 1.76; SIN-1, 3.13 ± 0.97, Fig. 3B). SIN-1 also decreased the insulin-stimulated activation of IRS-1-associated p85, a

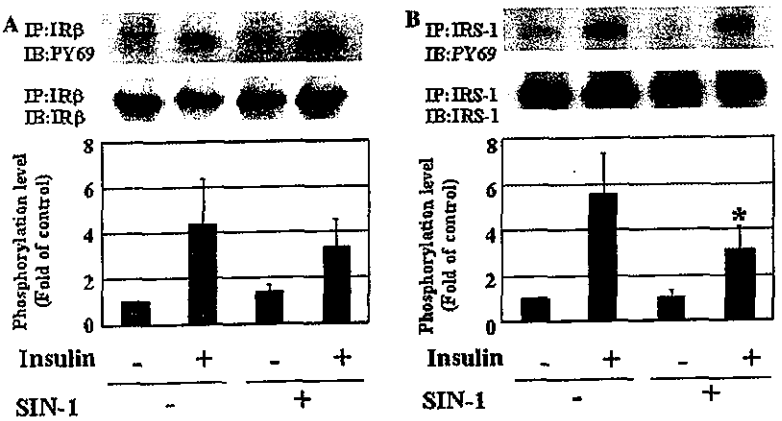

Fig. 3. Effects of peroxynitrite on phosphorylation of the insulin receptor and IRS-1. 3T3-L1 adipocytes were serum-starved and incubated with or without 4 mM SIN-1 for 12 h. Cells were stimulated with 100 nM insulin for 5 min. The cell lysates were immunoprecipitated with anti-insulin receptor β antibody (A) or anti-IRS-1 antibody (B), and then immunoblotted with anti-phosphotyrosine antibody (PY 69) (A,B). Results are from three independent experiments. The intensity of each band was quantitated relative to the band for cells incubated in the absence of insulin and SIN-1. Data are means ± SEM. Statistical significance was determined by Student's t test. *$p < 0.05$, compared with insulin-stimulated control cells. All blots were stripped and then reprobed with anti-insulin receptor β antibody (A) or anti-IRS-1 antibody (B) to confirm that similar amounts of proteins were applied to each lane.

regulatory subunit of PI-3 kinase reducing it by 76% (Control, 9.29 ± 2.04; SIN-1 2.60 ± 0.61, Fig. 4A). In agreement with the Western blotting data, insulin-stimulated IRS-1-associated PI3-kinase activity was also markedly decreased in SIN-1-treated 3T3-L1 adipocytes (Control 12.52 ± 4.15; SIN-1 3.18 ± 0.78, Fig. 4B). Reflecting the decrease of IRS-1-associated PI3-kinase activity, SIN-1 markedly decreased insulin-stimulated serine and threonine phosphorylation of Akt (Figs. 5A and B). On the other hand, SIN-1 only induced a modest decrease of the insulin-stimulated tyrosine/threonine-phosphorylation of Erk. The associated increase of basal Erk phosphorylation might be due to the effect of increased oxidative stress on the cells (Fig. 5C). While peroxynitrite decreased the level of IRS-1 protein, independent of this fall in the protein level, peroxynitrite also impaired the insulin-stimulated phosphorylation of IRS-1, i.e., peroxynitrite reduced glucose uptake by insulin-stimulated 3T3-L1 adipocytes through impairment of downstream signals.

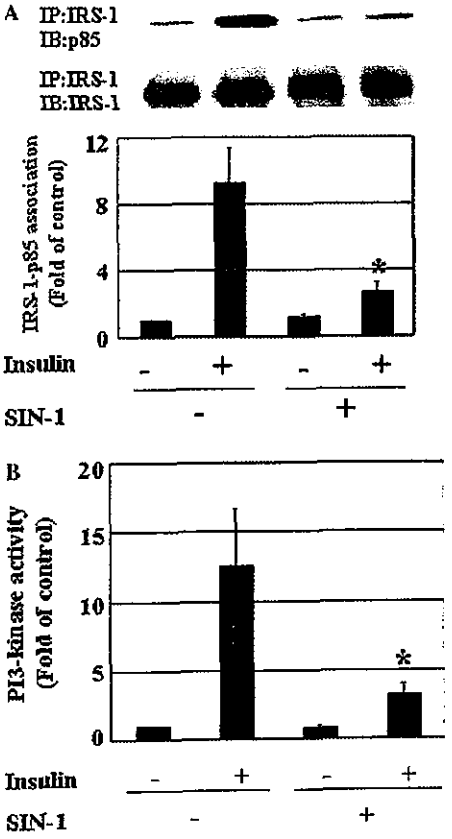

Fig. 4. Peroxynitrite impairs IRS-1-associated PI3-kinase activity. (A) 3T3-L1 adipocytes were incubated in serum-free medium with or without 4 mM SIN-1 for 12 h. Cells were stimulated with 100 nM insulin for 20 min. Cell lysates were immunoprecipitated with anti-IRS-1 antibody and then immunoblotted with anti-p85 antibody. Results are from at least three independent experiments. The intensity of each band was quantitated relative to the band for cells incubated in the absence of insulin and SIN-1. Data are means ± SEM. All blots were stripped and reprobed with anti-IRS-1 antibody to confirm that similar amounts of proteins were applied to each lane. (B) 3T3-L1 adipocytes were incubated in serum-free medium with or without 4 mM SIN-1 for 12 h and then stimulated with 100 nM insulin for 20 min. The cell lysates were immunoprecipitated with anti-IRS-1 antibody. Experiments were performed in triplicate on three separate occasions. The immunoprecipitates were subjected to PI3-kinase assay using phosphatidylinositol as a substrate. The intensity of each band was quantitated relative to that of cells incubated in the absence of insulin and SIN-1. Data are means ± SEM. Statistical significance was determined by Student's t test. $^{*}p < 0.05$ compared with insulin-stimulated control cells.

Peroxynitrite induces tyrosine nitration of IRS-1 but not Ser^{307}-phosphorylation of IRS-1

To further investigate the mechanism(s) of the effects of peroxynitrite on insulin signalling, we assessed whether treatment with SIN-1 could induce phosphorylation of the Ser^{307} residues of IRS-1. Ser^{307} phosphorylation of IRS-1 was detected in TNF-α-treated cells but not in SIN-1-treated cells (Fig. 6A). These results demonstrated that phosphorylation of the Ser^{307} residues of IRS-1 is not involved in the impairment of insulin signalling by peroxynitrite. Peroxynitrite is known to induce the nitration of Tyr of several molecules. Thus, we investigated tyrosine nitration of IRS-1 in SIN-1-treated 3T3-L1 adipocytes. In 3T3-L1 adipocytes, only a modest tyrosine nitration of IRS-1 could be detected (data not shown). Therefore, we investigated tyrosine nitration of IRS-1 in SIN-1-treated HIRc. Different from 3T3-L1 adipocytes, SIN-1-treated HIRc does not show the decrease in the level of IRS-1 protein (Fig. 6B). In these cells, Ser^{307} phosphorylation of IRS-1 was also not detected, but tyrosine nitration of IRS-1 was detected in both 4 and 8 mM SIN-1 (Fig. 6C). In TNF-α-treated cells, where we found the induction of iNOS expression (data not shown), both Ser^{307} phosphorylation and tyrosine nitration of IRS-1 were detected (Fig. 6C).

Detection of nitrated tyrosine residue of peroxynitrite-treated IRS-1

To investigate the relation between peroxynitrite-induced tyrosine nitration of IRS-1 and impairment of insulin signal pathway, recombinant rat IRS-1 was incubated with 50 μM peroxynitrite for 1 h. As shown in Fig. 7A, tyrosine nitration of rat IRS-1 was clearly detected by Western blotting (an approximately 150 kDa band). Next, we investigated the nitrated tyrosine residues of IRS-1 after peroxynitrite treatment by MS. After in-gel digestion of the band stained with CBB, peptide mapping was carried out. A total of 25 trypsinized peptides of IRS-1 were identified by LC-MS and the PROWL search engine. The amino acids covered 31.5% of the whole sequence of IRS-1 (389/1235). The amino acid sequences of six peptides were also

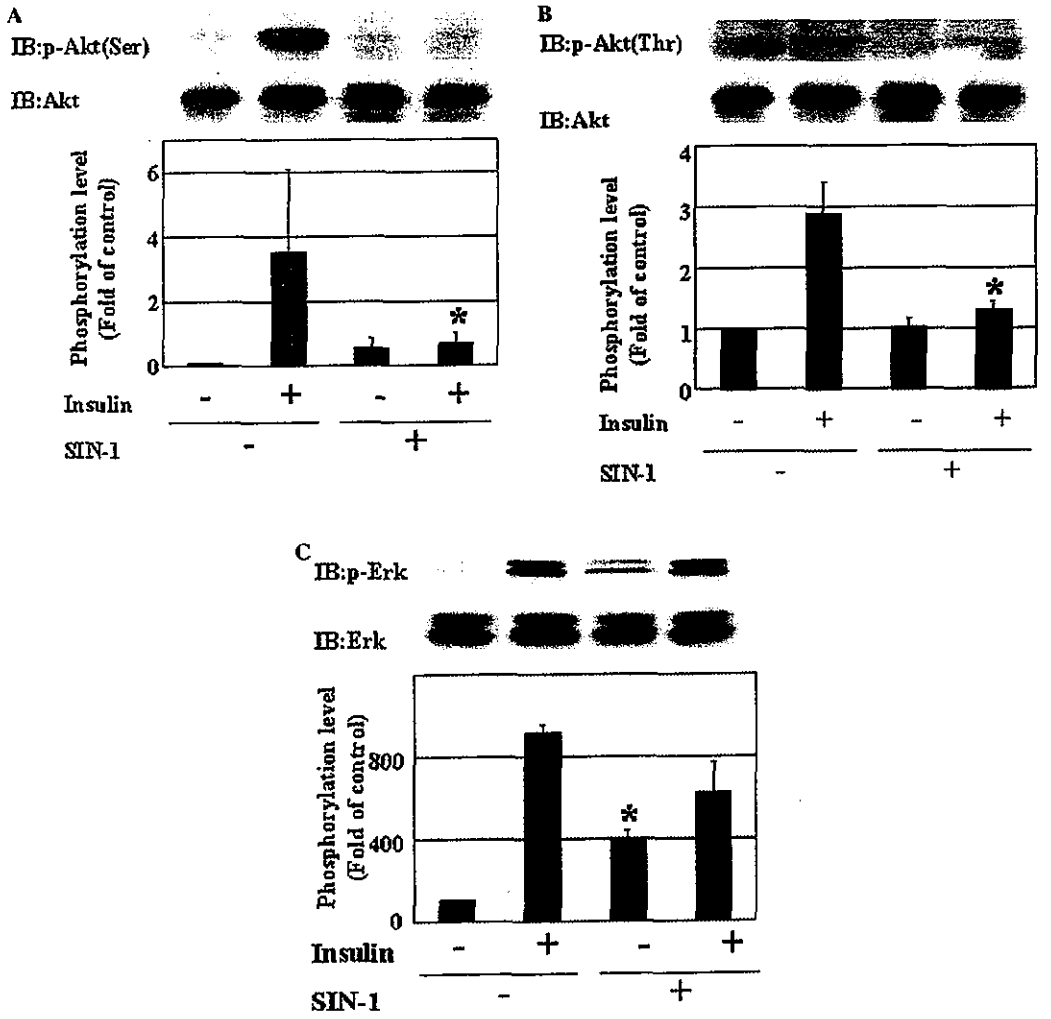

Fig. 5. Effects of peroxynitrite on phosphorylation of Akt and Erk. Differentiated 3T3-L1 adipocytes were incubated in serum-free medium with or without 4 mM SIN-1 for 12 h. Cells were stimulated with 100 nM insulin for 20 min and then immunoblotted with anti-serine (473) phosphospecific Akt antibody (A), anti-threonine (308) phosphospecific Akt antibody (B), and anti-phosphospecific Erk antibody (C). Results are from three independent experiments. The intensity of each band was quantitated relative to the band for cells incubated in the absence of insulin and SIN-1. Data are means ± SEM. Statistical significance was determined by Student's t test. $^*p < 0.05$ compared with insulin-stimulated control cells. The bands were stripped and reprobed with anti-Akt antibody (A,B) or anti-Erk antibody (C).

confirmed by LC-MS/MS and the MASCOT search engine (6.2% of IRS-1) (Table 1). We found four unique peptides, the tyrosine of which residues was nitrated by peroxynitrite. These were detected by SIM of peptide mapping at the ion of $[M+2H]^{2+}$ and $[M+45+2H]^{2+}$ because increase of 45 Da corresponded to the NO_2 group. Modified peptides also appeared at 30–150 s behind the unmodified protein (Fig. 7B). The four peptides at residues 489–515 (Tyr^{489}), 932–948 (Tyr^{939}), 997–1014 (Tyr^{999} or Tyr^{1010}), and 1169–1179 (Tyr^{1172}) were identified as peptides containing nitrated tyrosine (Table 1). The ratio of modified to unmodified peptides was 4.1% at Tyr^{489}, 26.9% at Tyr^{939}, 8.0% at Tyr^{999} or Tyr^{1010}, and 35.3% at Tyr^{1172} (Table 1). The residues at Tyr^{939} and Tyr^{1172} were identified as the phosphorylation site of IRS-1 according to the SWISS-PROT database and seemed to be more easily nitrated than Tyr^{489} and Tyr^{999} or Tyr^{1010}. These results demonstrate that at least two of the phosphorylation sites of the tyrosine residues of IRS-1 could be nitrated by exposure to peroxynitrite.

Discussion

Insulin receptor substrate (IRS) proteins are essential signalling molecules that mediate the metabolic actions of insulin and, IRS-1 and -2 are recognized as especially important molecules in glucose metabolism [18]. The activated insulin receptor-related tyrosine kinase phosphorylates multiple tyrosine residues of