Nitric oxide synthase expression and activity in the tight-skin mouse model of fibrosis

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Objectives. Nitric oxide (‘NO) is an important physiological signalling molecule and a potent vasodilator. We have previously demonstrated abnormal ‘NO metabolism in the plasma of patients with systemic sclerosis (SSc; scleroderma), a disease that features vascular dysfunction as well as collagen overproduction and fibrosis. The aim of the present study was to examine nitric oxide synthase (NOS) expression and activity and assess the potential role of antioxidants in the scleroderma-like syndrome of the tight-skin 1 (TSK-1/⁺) mouse, an experimental animal model for fibrosis.

Methods. Skin, lung or plasma was taken from TSK-1/⁺ (n = 15) and wild-type (WT; n = 12) littermate mice. Type 1 collagen, endothelial nitric oxide synthase (eNOS), haemoxygenase-1 (HO-1) and nuclear factor erythroid 2-related factor 2 (Nrf2) protein and gene expression were determined by western blot and reverse transcriptase-polymerase chain reaction. eNOS expression was further determined by immunohistochemistry.

Results. In the skin, eNOS was present in the epidermal layer, hair follicles and also in the endothelial cells lining the blood vessels. Expression of both the eNOS protein and gene was significantly reduced in TSK-1/⁺ skin tissue, while type 1 collagen protein was elevated compared with WT. Furthermore, there was decreased NOS activity in TSK-1/⁺ skin tissue; however, there was no measurable difference in plasma NOx. Correspondingly, the protective antioxidant enzyme HO-1 and the associated transcription factor Nrf2 showed decreased protein and gene expression levels in TSK-1/⁺ skin, while there was also less total antioxidant activity. In TSK-1/⁺ lung tissue, however, we observed no difference in collagen protein expression, ‘NO metabolism or HO-1 expression and total antioxidant activity compared with WT.

Conclusions. The findings suggest that there is also abnormal ‘NO metabolism in the TSK-1/⁺ mouse model of fibrosis, particularly in the skin, while expression and activity of protective antioxidants are reduced. The TSK-1/⁺ mouse may also be useful for testing treatments that target vascular endothelial cell function in patients with SSc.

Key words: Nitric oxide, TSK-1 mice, Fibrosis, Systemic sclerosis, Scleroderma.

Introduction

Systemic sclerosis (SSc; scleroderma) is a generalized connective tissue disorder characterized by an increased synthesis and deposition of collagen and other extracellular matrix proteins. Progressive fibrosis of the skin and internal organs such as the lungs, heart, gastrointestinal tract or kidneys is evident, along with microvascular dysfunction, inflammatory and immunological complications [1, 2]. Endothelial activation and damage is a key part of this process [3, 4] along with the associated Raynaud’s phenomenon (RP), which is distinguished by cold-induced digital ischaemic attacks and vasospasms [5]. Although cell signalling molecules such as the vasoconstrictor endothelin [6] and the vasodilator nitric oxide (‘NO) [7–10] reflect the vasculopathy of SSc, the pathogenesis of SSc is still unknown.

The tight-skin (TSK) mouse, an experimental model for the fibrotic elements of SSc, is valuable for studying the pathological mechanisms involved in this disease process and testing potential therapeutic treatments [11, 12]. TSK mice have an autosomal dominant mutation localized to chromosome 2 of the fibrillin-1 gene. Mice homozygous (TSK/TSK) for the mutation die in utero by 8–10 days of gestation, while heterozygous (TSK/⁺) mice develop a scleroderma-like syndrome. Type-1 TSK (TSK-1/⁺) mice characteristically exhibit marked thickening of subcutaneous dermal tissue; fibrotic abnormalities of the heart; features of SSc autoimmunity such as positivity for RNA polymerase I, anti-Sc/70 or antinuclear antibodies; and distended emphysematous lungs with little fibrosis. However, the mice are considered to lack the inflammatory mononuclear cell infiltration of affected organs and severe histological vascular involvement of SSc [12–17].

‘NO is a free radical synthesized from l-arginine by an inducible expression (iNOS or NOS 2) in response to a variety of stimuli is possible, with ‘NO-mediated signalling apparent in the skin [19]. At present, only a few studies have examined endothelial involvement or ‘NO metabolism (seen as a reflection of endothelial dysfunction in many diseases) in the TSK-1/⁺ mouse. Endothelial dysfunction has been shown in the thoracic aorta of TSK-1/⁺ mice [20], while impaired endothelium-dependent vasodilation, attenuated angiogenic responses to vascular endothelial growth factor and increased plasma levels of angiotatin and pro-inflammatory high-density lipoprotein have recently been reported in TSK-1/⁺ mice [21].

The suggestion that ‘NO and other reactive oxygen species such as ONOO⁻ and O₂⁻ are implicated in the pathogenesis of SSc has been supported by studies showing evidence of ‘NO overproduction in scleroderma skin and fibroblasts [8, 9], increased levels of O₂⁻ [22] and increased circulatory levels of nitrotyrosine, a marker for peroxynitrite production [23, 40]. In addition, we have reported abnormal ‘NO metabolism in plasma
from SSc patients, as well as the loss of NO-induced inhibition of type 1 collagen synthesis in dermal SSc fibroblasts [23, 24]. Furthermore, we have demonstrated in our earlier work that there are low levels of plasma antioxidants such as ascorbic acid in scleroderma [25], while others have reported the beneficial effects of antioxidant therapy in SSc and the associated RP [26–28]. Endogenous antioxidant defence enzymes are also important mechanisms by which cells limit the damage caused by oxygen free radicals. For example, the induction of haemoxenogense-1 (HO-1), and its associated transcriptional factor, nuclear factor erythroid 2-related factor 2 (Nrf2), is considered to enhance the antioxidant activity of cells and provide protection under conditions of increased oxidative stress [29, 30].

The aim of the present study was to examine NOS expression and activity and to assess the potential role of antioxidants in the scleroderma-like syndrome of the TSK-1/+ mouse.

Materials and methods

Mice

All animal experiments were carried out according to the guidelines of the Royal Free Hampstead NHS Trust local research ethics committee, who also gave ethical approval for the study. Fifteen adult (3–4 months old) TSK-1/+ mice (coat colour: black) of both sexes were used. Twelve wild-type (WT) (coat colour: pallid) littermate mice served as normal controls, as previously described [31]. TSK-1/+ and WT mice were sacrificed under a CO₂ atmosphere and skin (interscapular region) or lungs were removed and the portions were snap-frozen (liquid N₂). Blood samples were also obtained and in order to minimize the effect of the diet on the plasma measurement of endogenously generated nitrite and nitrate, mice were fed a semi-synthetic diet containing low levels of nitrate and nitrite for 10 days prior to the collection of blood. All blood samples were kept on ice, and the plasma fraction (1000 g at 4°C for 10 min) was obtained 15 min after sampling.

Western blot analysis of type 1 collagen, eNOS, HO-1 and Nrf2

Mouse skin or lung tissue was homogenized in lysis buffer [1 mmol/l Ethylenediaminetetraacetate (EDTA), 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate, 1% Triton, 1 mM phenylmethylsulphonyl fluoride, 1 mmol/l NaF, 1 mmol/l Na orthovanadate, 1 µg/ml aprotinin, 5 µg/ml leupeptin and 5 µg/ml pepstatin]. The samples were centrifuged at 10 000 g for 5 min and the supernatant was separated from the pellet. Protein concentrations of the supernatant samples were determined using the bicinchoninic acid (BCA) protein assay (Pierce, Illinois, USA). For all gels, 50 µg of sample protein was run on a NuPAGE 4–12% gradient gel (Invitrogen, Paisley, USA) under reducing conditions. Proteins were transferred onto nitrocellulose membranes and incubated in a blocking buffer (5% non-fat dried milk, 0.1% Tween-20) for 1 h. Briefly, the membranes were incubated overnight with an anti-collagen type 1 polyclonal antibody (1:500; Southern Biotechnology Associates, Alabama, USA), anti-eNOS polyclonal antibody (1:500; Transduction Laboratories, KY, USA), anti-haemoxenogenase 1 polyclonal antibody (1:500; Abcam, Cambridge, UK), anti-Nrf2 polyclonal antibody (1:750; Santa Cruz, California, USA) or additionally anti-β-actin polyclonal antibody (1:500; Abcam). The membranes were probed with the appropriate horseradish peroxidase-conjugated secondary antibody with detection by West Dura chemiluminescent substrates (Pierce) and visualized using a camera imager (Alpha Innotech, California, USA) and arbitrary relative density units (RDUs) were recorded.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) of eNOS, HO-1 and Nrf2

Mouse skin or lung tissue was homogenized in RLT lysis buffer (Qiagen Ltd, West Sussex, UK). The QiAamp RNA (Qiagen Ltd) mini protocol for isolation of total RNA from tissues was used according to the manufacturer’s instructions. The concentration and purity of RNA were determined so that 1 µg (lun) or 0.5 µg (skin) of RNA could be used to start RT-PCR.

RT-PCR was performed using the one-step RT-PCR Kit (Qiagen Ltd). Primers specific for mouse eNOS 459 bp ([CTG GAC ACC AGG ACA ACC- (forward) and GCT GCT GTG CGT AGC TCT- (reverse)], mouse HO-1 313 bp [TCC CAG ACA CCG CTC CTC CAG- (forward) and GGA TTT GGG GCT GGT GTT TTC- (reverse)], mouse Nrf2 217 bp [AAG CCC CAT TCA CAA AAG AC- (forward) and GCG ACT TTA TTC TTA CCT CTC C- (reverse)] and mouse β-actin 513 bp [TGT GAT GGT GGG AAA GAT TCA- (forward) and TTT GAT GTC ACG CAC GAT TTC C- (reverse)] were used. The thermal cycling conditions were as follows: 1 × (50°C, 30 min), 1 × (95°C, 15 min), then n cycles of 94°C, 1 min, x°C, 1 min and 72°C, 1 min, followed by 1 × (72°C, 10 min). The annealing temperature (x) for all the primers was 60°C. The number of cycles (n) for each set of primers was 35, 28, 28 and 28, respectively. PCR products were resolved by agarose gel (1%) electrophoresis and stained with ethidium bromide. The products were then visualized under UV light using a camera imager (Alpha Innotech) and arbitrary RDUs recorded. Data were normalized to the β-actin signal intensity.

Histology and eNOS immunohistochemistry

Mouse skin or lung tissue was formalin fixed and embedded in paraffin. Sections (5 µm thick) were cut, mounted on glass slides, deparaffinized, rehydrated in graded alcohol solutions and stained with haematoxylin and eosin for histological assessment. For immunohistochemistry, lung sections were further subjected to antigen retrieval by heating sections to 95°C in 0.01 mol/l citrate buffer (pH 6.0) for 20 min and slowly cooling to room temperature. Briefly, skin or lung sections were incubated for 15 min with 3% H₂O₂ to quench endogenous peroxidase activity. After blocking of non-specific sites with 5% goat serum in phosphate buffered saline (20 min), sections were incubated overnight (4°C) with a rabbit polyclonal antibody against eNOS (1:50 Transduction Laboratories, Kentucky, USA) and treated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Peterborough, UK) for 60 min at room temperature. The detection system was the Vectastain ABC-HRP kit (Vector Laboratories) with ImmPACT diaminobenzidine (Vector Laboratories) as the chromogenic substrate. Sections were counterstained with haematoxylin. Negative controls were carried out by omitting the primary antibody and using normal rabbit IgG.

NOS enzyme activity assay

Mouse skin or lung tissue was homogenized in ice-cold lysis buffer [25 mmol/l Tris–HCl, pH 7.4, 1 mmol/l EDTA, 1 mmol/l ethyleneglycoltetraacetic acid (EGTA)], subject to a freeze–thaw cycle and then spun for 5 min at 10 000 g and the lysate removed. Total NOS activity was determined by the conversion of [¹⁴C] L-arginine (Amersham Biosciences, Buckinghamshire, UK) to [¹⁴C] L-citrulline using a commercially available NOS activity assay kit (Calbiochem, Nottingham, UK). In brief, lysates were incubated in the presence or absence of 1 mM N⁶-nitroarginine methyl ester (L-NAME, 100 µmol/l) with a reaction buffer [25 mmol/l Tris–HCl, pH 7.4, 3 µmol/l tetrahydrobiopterin, 1 µmol/l flavine adenine dinucleotide (FAD), 1 µmol/l flavin mononucleotide, 1 mmol/l NADPH, 0.05 µg [¹⁴C]-arginine (~110 000 c.p.m.), 3 mmol/l CaCl₂ and water] and incubated at 37°C for 60 min. The reaction was then stopped by the addition of ice-cold 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (50 mmol/l,
pH 5.5), EDTA (5 mmol/l) and equilibrated resin to each sample. [14C]-citrulline was eluted from the samples and radioactivity quantified in a liquid scintillation counter. Protein concentrations of tissue lysates were determined by the BCA protein assay (Pierce) and retrieved [14C] t-citrulline values were normalized to total protein (picomoles/minute/milligram protein).

**Measurement of total plasma nitrate and nitrite (NOX)**

NOX is the sum of nitrate (NO\(_3\)) and nitrite (NO\(_2\)) anions. Concentrations of NO\(_2\) were measured in plasma by a *NO chemiluminescence detector (NOA 280; Sievers, Colorado, USA). Briefly, mouse plasma samples (1:5 dilution) were filtered through Ultrafree-4 filters and centrifuged for 1 h at 2500 g. After removing high molecular proteins, plasma samples, together with NO\(_3\) standard solutions, were incubated with Tris buffer (20 mmol/l, pH 7.6) containing nitrate reductase 10 mU, NADPH 40 μmol/l and FAD 1 μmol/l, for 1 h at 37°C to convert NO\(_3\) into NO\(_2\). Samples now containing NO\(_2\) were then reduced to nitric oxide in a purge vessel containing potassium iodide (1% in acetic acid) as previously described [23] to determine NO\(_2\).

**Measurement of total antioxidant activity**

Mouse skin or lung tissue was homogenized in potassium phosphate buffer (5 mmol/l, pH 7.4) containing 0.9% sodium chloride and 0.1% glucose. The samples were centrifuged at 10,000 g for 5 min and the supernatant was separated from the pellet. Total antioxidant activity was measured in the tissue supernatants or plasma using a commercially available total antioxidant assay kit (Sigma, Poole, UK), according to the manufacturer’s instructions. In brief, the assay relies on the ability of antioxidants in the sample to inhibit oxidation by metmyoglobin of 2,2’ azino-bis-[3-ethylbenz-thiazoline-6-sulphonic acid] (ABTS) to ABTS\(^+\). The amount of ABTS\(^+\) produced is monitored by reading the absorbance at 405 nm. Under these reaction conditions, the antioxidants in the sample cause proportional to their concentration. The capacity of the antioxidants in the sample to prevent ABTS oxidation is compared with that of a control antioxidant, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid [Trolox (Sigma), 0.4 mmol/l], a water-soluble tocopherol analogue. ABTS\(^+\) values were normalized to total protein in tissue supernatants.

**Data analysis**

Data were expressed as mean ± s.e.m., where n was the number of individual samples assayed in duplicate or triplicate. Data were analysed for statistical significance by unpaired Student’s t-test or comparisons of multiple means were made using one-way analysis of variance followed by the Student Newman–Keuls test. P < 0.05 was considered statistically significant.

**Results**

**Western blot analysis of type I collagen, eNOS, HO-1 and Nrf2 proteins in TSK-1/+ mouse skin and lung**

Collagens are the major components of fibrotic tissue. Western blot analysis was performed to assess the contribution of type I collagen protein expression to fibrosis in the skin and lung tissue of TSK-1/+ mice. The level of type I collagen immunoreactivity in TSK-1/+ skin supernatants was significantly greater than that observed for WT littermate controls (mean ± s.e.m. RDU\(^+\) 252.14 ± 67.97 in TSK-1/+ vs 108.50 ± 35.77 in WT; P < 0.05); however, no significant differences were observed in the lung supernatants of WT and TSK-1/+ mice (Fig. 1A and 1B).

TSK-1/+ mice also featured significantly decreased eNOS protein expression (RDU 18.21 ± 2.40 vs WT: 44.54 ± 12.52; P < 0.05) in skin supernatants with no difference occurring in the lung (Fig. 1A and C).

Protein expression of the antioxidant enzyme HO-1 and its associated transcription factor Nrf2 was also down-regulated in the TSK-1/+ mice skin compared with WT controls (HO-1 RDU 86.96 ± 22.77 vs WT: 190.86 ± 53.32; P < 0.05) and also (Nrf2 RDU 76.43 ± 9.57 vs WT: 177.34 ± 29.09; P < 0.05) as shown in Fig. 1A, D and E. No differences were observed in the lung in WT and TSK-1/+ mice (Fig. 1A, D and E).

**Gene expression analysis of eNOS, HO-1 and Nrf2 in TSK-1/+ mouse skin and lung**

To assess whether changes in eNOS, HO-1 and Nrf2 protein in mouse TSK-1/+ skin were also reflected at the gene level, mRNA gene expression was assessed using RT-PCR. eNOS mRNA gene expression was significantly decreased in the skin supernatants of TSK-1/+ mouse when compared with WT littermate controls (RDU 21.20 ± 6.45 vs WT: 61.46 ± 16.67; P < 0.05); however, no significant differences were observed in the lung WT and TSK-1/+ mouse supernatants (Fig. 2A). Similarly, HO-1 mRNA gene expression was also down-regulated in TSK-1/+ mouse skin compared with WT controls (RDU 73.04 ± 9.40 vs WT: 105.22 ± 12.87; P < 0.05) (Fig. 2B), while Nrf2 mRNA gene expression in TSK-1/+ mouse skin also showed an apparent decrease compared with controls, but did not reach statistical significance (Fig. 2C). Consistent with the data on protein expression, no differences were observed in HO-1 or Nrf2 mRNA gene expression in the lung tissue of WT and TSK-1/+ mouse (Fig. 2B and C).

**Immunohistochemistry for eNOS in TSK-1/+ mouse skin and lung**

The skin of TSK-1/+ mouse exhibit thickening of dermal tissue [14, 16, 17] compared with WT controls, as shown by haematoxylin–eosin staining (Fig. 3A and B). We sought to further confirm eNOS expression and localization in TSK-1/+ mouse skin by immunohistochemistry. Immunostaining for eNOS was present particularly in the hair follicles (Fig. 3D), epidermal layer (Fig. 3E) and also endothelial cells lining blood vessels (Fig. 3F and G) in both the TSK-1/+ mouse skin and WT controls. Non-immune rabbit IgG control showed no detectable staining (Fig. 3C). This observation is in agreement with studies showing eNOS expression in normal human epidermal cells, hair follicles and eccrine secretory glands of the dermis [32]. In skin sections from scleroderma patients, eNOS expression in capillary and venular endothelial cells has been reported to decrease as the skin lesion progresses into an actively fibrotic phase [8].

TSK-1/+ mice typically feature distended emphysematous lungs with little fibrosis and alveolar airspace enlargement [15, 33–35] compared with WT controls, as shown by haematoxylin–eosin staining (Fig. 3H and I). In the mouse lung, strong immunostaining for eNOS was detected in the parenchyma, particularly in type II cells of the alveoli (Fig. 3K) and in the bronchiolar epithelia (Fig. 3L) as well as endothelial cells lining the blood vessels (Fig. 3M) in both the TSK-1/+ mouse and WT controls, whereas little staining was observed in non-immune rabbit IgG controls (Fig. 3J).

**NOS activity, NO\(_x\) production and total antioxidant activity in TSK-1/+ mouse skin, lung and plasma**

Since there were abnormalities in nitric oxide protein and gene expression from the skin of TSK-1/+ mouse, we further measured the relative levels of NOS enzyme activity using a citrulline assay. NOS activity was reduced in the skin supernatants of TSK-1/+ mouse compared with WT controls. The synthetic NOS inhibitor
L-NAME (100 μmol/l) decreased NOS activity by only 29.8 ± 7.9% in skin TSK-1/+ compared with 55.0 ± 10.5% in WT controls (P < 0.05) (Fig. 4A). There were no significant differences observed in NOS activity between lung TSK-1/+ and WT control tissue (Fig. 4B).

In most biological situations, *NO is largely oxidized to nitrate (NO$_3^-$) and nitrite (NO$_2^-$), with the measurement of the total sum of nitrate and nitrite (NO$_x^-$) production seen as an index of endothelial dysfunction in many diseases. Thus, the levels of circulating NO$_x^-$ were also assessed in plasma from TSK-1/+ and WT controls. The mean values of each group were, however, not significantly different (58.9 ± 5.3 μmol/l in TSK-1/+ vs WT: 59.0 ± 3.8 μmol/l) (Fig. 4C).

Total antioxidant activity was also measured in TSK-1/+ mouse skin and lung supernatants, as well as plasma by inhibition of ABTS$^+$ production. ABTS$^+$ production was significantly greater by 44.9 ± 18.0% (P < 0.05) in TSK-1/+ mouse skin supernatants compared with WT controls, indicating less total antioxidant activity (Fig. 4D). There were no significant differences observed in ABTS$^+$ production between TSK-1/+ and WT controls in lung
tissue or plasma (Fig. 4D). The control antioxidant, Trolox (a water-soluble tocopherol analogue, 0.4 mmol/l), significantly inhibited ABTS*+ production by up to 81.1 ± 6.1% (P < 0.005) compared with controls (Fig. 4D).

Discussion

The present study was undertaken to investigate whether nitric oxide metabolism, and also antioxidants, plays a potential role in the pathogenesis of the scleroderma-like syndrome in the TSK-1/+ mouse. The results show that while collagen protein expression was elevated in TSK-1/+ skin tissue, eNOS protein and gene expressions were reduced compared with WT controls. In mouse skin, eNOS immunoreactivity was present in the epidermal layer, hair follicles and also endothelial cells lining the blood vessels. Furthermore, there was decreased NOS activity in TSK-1/+ skin tissue; however, there was no difference in circulating levels of *NO. Correspondingly, the protective antioxidant enzyme HO-1 and the associated transcription factor Nrf2 showed reduced protein and gene expression levels in TSK-1/+ skin, while there was also less total antioxidant activity. In the TSK-1/+ lung tissue, however, we observed no difference in collagen protein expression, *NO metabolism or antioxidant expression/activity. The findings suggest that there is also abnormal *NO metabolism in the scleroderma-like syndrome of the TSK-1/+ mouse, particularly in the skin, while expression and activity of protective antioxidants are reduced.

The TSK-1/+ mouse model of SSc has been shown to be a valuable tool in studying the pathological mechanisms involved in the disease and in testing potential therapeutic treatments [11, 12]. In particular, many groups have reported increased type I collagen production in the skin [36], similar to that found in SSc [37], with the TSK-1/+ mouse also characteristically featuring increased fibrosis and deposition of extracellular matrix components in the dermal tissue [14, 16, 17]. Our data also showed evidence of increased type I collagen protein expression and fibrosis in TSK-1/+ skin; however, no change in type I collagen protein was observed in the lung tissue. These results are not surprising because while in another study increased lung collagen synthesis was shown from 2 months of age [33], the lungs of TSK-1/+ mouse develop emphysema and not fibrosis, implying abnormal collagen remodelling [15]. Indeed, it has been reported that collagen turnover and degradation in the lungs is accelerated by alveolar macrophages [34], which results in remodelling of the alveolar collagen network in the lungs of TSK-1/+ mouse [35]. In contrast, in SSc patients, pulmonary involvement, either due to parenchymal fibrosis and/or pulmonary arterial hypertension, is a major cause of morbidity and mortality [1]. Nevertheless, the TSK-1/+ mouse model of fibrosis still serves as a useful model in investigating the pathogenic components involved in scleroderma, particularly mechanisms involved in skin fibrosis [11, 12, 31, 38].

Nitric oxide has been shown to play a crucial role in control of vascular tone and impairment in constitutive *NO expression could reflect the integrity of the vascular endothelium [39]. In SSc, reduced eNOS expression and microcirculatory dysfunction is in part contributory to the associated RP that is well described in these patients [2–5, 8]. Indeed, many studies have reported evidence of abnormal *NO metabolism in scleroderma patients [7–9, 23, 40] and furthermore, recently, several laboratories have proposed using therapies that target vascular endothelial cell function to treat patients with SSc [41, 42]. In the present study, a significantly decreased eNOS protein and gene expression was shown in TSK-1/+ skin tissue; however, this was not reflected in lung tissue, which was similar to that in controls. Furthermore, NOS activity was also significantly decreased in TSK-1/+ skin. This is a novel finding that is consistent with the study where Marie and Beny [20] demonstrated endothelium dysfunction in response to endothelium-dependent vasodilators in the thoracic aorta of TSK-1/+ mouse. Additionally, impaired

FIG. 2. Gene expression levels of eNOS, HO-1 and Nrf2 in TSK-1/+ mouse skin and lung determined by RT-PCR. Skin or lung samples of TSK-1/+ and WT mice were processed for RT-PCR analyses, as described in the Materials and methods section. Digitized images of mRNA bands on agarose gels were quantitated and normalized to an internal control gene (β-actin). (A) RT-PCR analyses of eNOS from skin (TSK-1/+, n = 8; WT, n = 8) and lung (TSK-1/+, n = 9; WT, n = 9) supernatants. (B) RT-PCR analyses of HO-1 from skin (TSK-1/+, n = 4; WT, n = 4) and lung (TSK-1/+, n = 4; WT, n = 4) supernatants. (C) RT-PCR analyses of Nrf2 from skin (TSK-1/+, n = 8; WT, n = 4) and lung (TSK-1/+, n = 5; WT, n = 5) supernatants. Data (mean ± S.E.M.) were expressed as RDUs. *P < 0.05 was considered statistically significant compared with WT.
endothelium-dependent vasodilation, increased oxidative stress and impaired angiogenesis have also recently been reported in the TSK-1/\(^+\) murine model of SSc [21]. The cause of endothelial impairment and injury in the TSK-1/\(^+\) mouse or in SSc patients remains unclear; however, elevated levels of reactive oxygen species, endothelial cell-specific autoantibodies, inflammatory cytokines, granzymes and vasculotropic viruses have been proposed [43, 44]. Combined with endothelial apoptosis, the process results in an abnormality and absence of blood vessels, particularly in SSc patients with late-stage disease. In this context,
it is important to note that Sgonc et al. [45] demonstrated that there is a lack of endothelial cell apoptosis in the dermis of TSK-1/+ mice, which may in part explain the absence in the mouse of the severe vascular involvement evident in SSc patients [12–16]. Nevertheless, our data further indicate the possibility that endothelial NO from blood vessels and restoration of endothelial impairment may be a crucial therapeutic target in skin fibrosis. Interestingly, with respect to other animal models of fibrosis, NO has been reported to act as a potent anti-fibrotic effector. Chung et al. [46] demonstrated that in mice after experimentally induced fibrosis, a loss of *NO bioactivity, by using eNOS knockout mouse, resulted in prolonged fibrosis. Furthermore, another study also demonstrated that overexpressing eNOS, using transgenic mouse, reduced fibrotic content after bleomycin-induced fibrosis [47]. In rats, the long-term inhibition of the inducible form of NOS (iNOS) has also been shown to favour the development of fibrosis [48].

Our data on plasma NOx production in TSK-1/+ mouse indicated no significant change in levels compared with controls. This may seem surprising, given our observation of decreased fibrosis, a loss of *NO bioactivity, by using eNOS knockout mouse, resulted in prolonged fibrosis. Furthermore, another study also demonstrated that overexpressing eNOS, using transgenic mouse, reduced fibrotic content after bleomycin-induced fibrosis [47]. In rats, the long-term inhibition of the inducible form of NOS (iNOS) has also been shown to favour the development of fibrosis [48].

Our data on plasma NOx production in TSK-1/+ mouse indicated no significant change in levels compared with controls. This may seem surprising, given our observation of decreased

![Image of graphs and tables showing NOS activity, NOx production, and total antioxidant activity in TSK-1/+ mouse skin, lung, and plasma.](image-url)
eNOS expression and activity in the TSK-1/+ skin; however, there were no observed changes in lung tissue. This may indicate that abnormal *NO metabolism is a localized occurrence in the skin, possibly involving small amounts of constitutive NO as opposed to eNOS in all the other unaffected tissues and the longer-lasting inducible form of the enzyme. Interestingly, in human SSC, several groups have measured plasma NOx production and apparent contradictory results have emerged, with both elevated and reduced levels reported [7, 9, 10]. The discrepancy in these results could be explained by differences in the degree of inflammation, disease subset and treatment of the patients. We have also previously reported elevated levels of circular nitrate/nitrite in the disease of primary RP and limited SSC, but not diffuse SSC [23].

Antioxidant enzymes are an important mechanism by which cells limit the damage caused by oxygen free radicals. In SSC, there is considerable evidence that free radicals and oxidative stress may be involved in the causation and perpetuation of tissue damage. Studies show evidence of *NO overproduction in scleroderma skin, fibroblasts and plasma [7–9, 23], increased levels of superoxide anions [22], antibodies against oxidized low-density lipoproteins [49], enhanced lipid peroxidation [50], increased F2-isoprostanes [51, 52] and increased circulatory levels of nitrotyrosine [23, 40]. In addition, it has been reported that there is reduced antioxidant capacity in SSC with, for example, ascorbic acid, α-tocopherol, β-carotene and selenium found to be lower in patients than in controls [25–27]. Subsequently, antioxidant therapy has been proposed as a possible treatment in SSC [26–28] and also in other diseases [53].

In the present study, the extent to which antioxidants play a potential role in the pathogenesis of the scleroderma-like syndrome in the TSK-1/+ mouse was also investigated. Our data indicate that, consistent with the findings on *NO metabolism, there was also lower total antioxidant activity in TSK-1/+ skin compared with controls, while no significant difference was observed in the lung tissue. Additionally, the results showed there was decreased protein and gene expression of the endogenous antioxidant enzyme HO-1 in TSK-1/+ skin compared with controls. HO-1 is induced by a variety of cellular stress, including oxygen deprivation, free radical-mediated stresses and *NO [54]. There is a large body of evidence suggesting that HO-1 is a cytoprotective enzyme and its induction in the setting of increased cellular stresses helps maintain physiological homeostasis [55]. Thus, embryonic fibroblasts derived from HO-1−/− knockout mice are significantly less resistant to the cytotoxicity induced by hydrogen peroxide and pararquat than WT controls [29, 30]. Conversely, cells overexpressing HO-1 have been reported to be more resistant to oxidant-induced toxicity than controls [29]. The HO-1-associated transcription factor Nr2f2 has also been shown to be a critical factor in protection against oxidative stress insults [56, 57], with Nr2f2−/− knockout mouse reported to show increased blemycin-induced pulmonary fibrosis [58] and expression of extracellular matrix genes such as collagens after hyperoxic exposure [57] when compared with WT controls. It has thus been suggested that an increased oxidative burden by suppression of antioxidant defence mechanisms in Nr2f2−/− mouse secondarily triggers regulation of extracellular matrix genes for repair responses [57]. In TSK-1/+ mouse, our findings of reduced HO-1 antioxidant protection and lower total antioxidant activity compared with WT controls, as well as decreased *NO metabolism, particularly in the skin, may contribute to the pathogenesis of the fibrosis. The scleroderma-like syndrome of the TSK-1/+ mouse may also be useful for testing treatments that target vascular endothelial cell function.

In conclusion, the findings suggest that there is also abnormal nitric oxide metabolism in the TSK-1/+ mouse model of fibrosis, particularly in the skin, while expression and activity of protective antioxidants are reduced. This suggests that in addition to fibrosis, the TSK-1/+ mouse may also exhibit vascular dysfunction as is the case for scleroderma.

Rheumatology key messages

- The findings suggest that there is abnormal nitric oxide metabolism in the TSK-1/+ mouse model of fibrosis, particularly in the skin.
- The scleroderma-like syndrome in TSK-1/+ mice may also be useful for testing treatments that target vascular endothelial cell function in scleroderma patients.

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