FEBS Letters 355 (1994) 178–182

FEBS 14814

The functional expression of p47-*phox* and p67-*phox* may contribute to the generation of superoxide by an NADPH oxidase-like system in human fibroblasts

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Received 10 October 1994

Abstract Recent evidence suggests that a number of non-phagocytic cell types may contain a superoxide generating NADPH oxidase. Studies to date on cultured human fibroblasts have primarily concerned the identification of cytochrome b_{558}, whilst expression of other NADPH oxidase components have not been addressed. In this study we have investigated the expression of NADPH oxidase with particular reference to the cytosolic factors p47-*phox* and p67-*phox*. Reverse transcriptase-polymerase chain reaction (RT-PCR) showed that human fibroblasts express mRNA for p47-*phox*, p67-*phox* and p22-*phox*. Expression of the gp91-*phox* transcript was not detected, indicating that human fibroblasts may possess an NADPH oxidase isoenzyme. Western blot analysis of human fibroblast cytosol, using an anti-p47-*phox* antibody (JW-1), identified a 47 kDa protein. Cell-free reconstitution assays showed that fibroblast cytosol could initiate superoxide generation when mixed with either human fibroblast membranes (0.16 nmol superoxide/min/μg membrane protein), or resting human neutrophil membranes (0.20 nmol superoxide/min/μg membrane protein). These data indicate that the expression of p47-*phox* and p67-*phox* by human fibroblasts may contribute to the cells' generation of superoxide.

Key words: NADPH oxidase; Superoxide; Human fibroblast

1. Introduction

The generation of superoxide is primarily associated with the microbicidal activity of phagocytic leukocytes, such as neutrophils and macrophages. In these cells, superoxide is produced by a multi-component enzyme system, which facilitates the reduction of oxygen to superoxide through the oxidation of cytosolic NADPH. This enzyme, known as the NADPH oxidase (see [1,2] for reviews), consists of a membrane-bound flavocytochrome *b* (cytochrome b_{558}), consisting of a small 22 kDa subunit (p22-*phox*) and a larger 90–110 kDa glycoprotein subunit (gp91-*phox*)[3]. The redox mid-point potential of cytochrome b_{558} ($E_{m,7}$ = −245 mV [4]) is sufficiently low to facilitate the single electron reduction of oxygen to superoxide ($E_{m,7}$ = −160 mV [5]). Sequence comparisons with known pyridine nucleotide binding proteins have suggested that gp91-*phox* may contain NADPH and FAD binding sites [6–8]. In addition to these membrane-bound components, a number of cytosolic factors (p47-*phox*, p67-*phox* and p21rac) are required for enzyme activity [9–11]. These components translocate to the membrane on activation where they initiate superoxide production [12]. Although precise functions for p47-*phox* and p67-*phox* have not yet been elucidated, their importance in the NADPH oxidase is clearly demonstrated by their absence in autosomal forms of the genetic disorder chronic granulomatous disease (CGD) (see [13] for review).

Recent studies have demonstrated that a number of non-phagocytic cell types, including fibroblasts [14], generate low levels of superoxide, often in response to specific stimuli (see [15] for review). Although numerous roles have been suggested for this production, both the source and function of non-phagocyte-derived superoxide remains unknown (see [16] for review). One possible source for the production of superoxide is an NADPH oxidase-like system. In particular, human fibroblasts [17] and human glomerular mesangial cells [18] have been shown to contain a membrane-bound *b*-type cytochrome with spectroscopic characteristics of cytochrome b_{558}. Furthermore, human glomerular mesangial cells express mRNA for p47-*phox* [19]. Additional work by Meier et al. [20] suggested that the cytochrome b_{558} of human fibroblasts was structurally and genetically distinct from the *b*-type cytochrome of the neutrophil NADPH oxidase. However, to date, no study has examined the expression of the cytosolic factors in human fibroblasts.

In this present communication we provide evidence which supports the presence of an NADPH oxidase-like system in human fibroblasts and demonstrate that these cells express mRNA for p22-*phox*, p47-*phox* and p67-*phox*. We also show the expression of p47-*phox* at the protein level in fibroblast cytosol and demonstrate that fibroblast cytosol can initiate superoxide generation in a heterologous cell-free reconstitution system. The functional expression of these components may contribute to the generation of superoxide by human fibroblasts.

2. Materials and methods

2.1. Culture of human fibroblasts

Human fibroblast cultures (kindly provided by Dr. V.B. O'Donnell, Rowett Research Institute, Aberdeen, Scotland) were obtained from individuals with Dupuytren's contracture. Cells were propagated in RPMI 1640 (Gibco-BRL) containing 10% (v/v) foetal calf serum (FCS) (Northumbria Biologicals Ltd.), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin (all supplied by Gibco-BRL). Fibroblast cultures were incubated at 37°C with a 5% CO_2 atmosphere and grew as a distinct adherent monolayer. Cells were passaged using trypsin/EDTA/glucose (0.125% w/v: 0.01% w/v: 0.1% w/v). All experiments were performed on cultured cells between the 6th and 10th passage.

2.2. Isolation of total RNA

Fibroblast cultures were washed in phosphate buffered saline (pH 7.4) and lysed by the addition of RNAzol B (Biogenesis Ltd.). Total RNA was subsequently isolated using the protocol outlined by the

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Abbreviations: RT-PCR, reverse transcriptase-polymerase chain reaction; *phox*, phagocytic oxidase (components of NADPH oxidase).

SSDI 0014-5793(94)01201-6

manufacturer of RNAzol B. RNA concentrations were determined spectroscopically by measuring the absorbance at 260 nm. The intregrity of the sample was assessed by measuring the 260 nm:280 nm ratio and by ethidium bromide staining of the RNA in a 2% (w/v) agarose gel (Ultrapure, BRL). Aliquots of total RNA extracts were stored at −70°C as precipitates in 50% ethanol containing 1.6 M ammonium acetate.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was reverse transcribed into cDNA, using the random hexanucleotide primer method previously described by O'Bryan et al. [21].

PCR amplification was performed in a Perkin Elmer thermocycler (Perkin Elmer-Cetus) using the method outlined by Topley et al. [22]. Differential PCR [23] was carried out with α-actin as a reference gene. Products of PCR amplification were separated on a 2% (w/v) NuSieve (Flowgen Ltd.), 1% (w/v) agarose gel containing ethidium bromide and visualised by UV transillumination.

Information regarding all the oligonucleotide primers used in this study is shown in Table 1. These oligonucleotide primers have previously been shown to generate correctly sized products in human monocytes, EBV-transformed B-lymphocytes and human glomerular mesangial cells (Jones et al., in press; Wood et al., submitted for publication). Sequencing of these DNA products showed that they share greater than 98% homology with their neutrophil counterparts.

2.4. Isolation of membranes and cytosol

Membranes and cytosol were isolated from sub-confluent human fibroblasts by differential centrifugation of cell lysates. Briefly, cultured fibroblasts were washed three times in 50 mM Tris, 1 mM EDTA (pH 7.5) containing 1 mM PMSF and 1 mM benzamidine (extraction buffer). Flasks were broken open and the adherent cells scraped into one corner with 300 μl of extraction buffer. Cell lysates were pooled from 15–20 T75 tissue culture flasks and sonicated on ice, with three 15–20 s 50W bursts. The sonicate was centrifuged (9,000 × *g* for 10 min at 4°C) to remove mitochondria, sub-cellular organelles and nuclei. The resultant supernatant was centrifuged at 100,000 × *g* for 45 min at 4°C. The supernatant containing the cytosolic proteins was collected and the plasma membrane pellet resuspended in 50 μl extraction buffer. Protein concentrations were determined by the method of Bradford [24].

To concentrate the fibroblast cytosol for Western blot analysis, proteins were precipitated on ice with 10% trichloroacetic acid (TCA). The protein precipitate was pelletted in a microfuge, washed twice in acetone and air dried. Proteins were resuspended in SDS-PAGE sample buffer and boiled for 2–3 min prior to electrophoresis.

Human neutrophils were isolated from buffy coats (supplied by the South West Regional Transfusion Service, Southmead Hospital, Bristol) as previously described by Segal and Jones [25]. Membrane and cytosol fractions were prepared from resting neutrophils by differential centrifugation of disrupted cells as described for fibroblasts. The supernatant, containing the cytosolic proteins, and the membrane pellet were suspended at approximately 4 mg/ml in PBS and stored at −80°C.

2.5. Western blot analysis

Cytosolic proteins derived from both resting neutrophils and fibroblasts were separated by SDS-PAGE in a 10% separating gel. Proteins were transferred to a nitrocellulose membrane by electroblotting at 100 V for 60 min on ice and probed with a polyclonal antibody (JW-1) raised against a C-terminal peptide of p47-*phox* (residues 367–390: PRPSADLILNRCSESTKRKLASAC). Western blots were performed using Enhanced Chemiluminescence (Amersham), as previously described by Gorlach et al. [26].

2.6. Cell-free reconstitution assay

Superoxide generation was assessed in a cell-free reconstitution system using the method of Bromberg and Pick [27]. Briefly, combinations of membrane (5 μg) and cytosol (100 μg) in PBS (pH 7.4), derived from either fibroblasts or resting neutrophils, were incubated for 5 min at room temperature in the presence of 15 μM GTPγ-S and 40 μM sodium arachidonate (both supplied by Sigma). This mixture was added to cytochrome *c* (type IV, Sigma) and the assay performed in a total volume of 1 ml. The final concentration of cytochrome *c* was 100 μM. NADPH (100 μM) was then added and the production of superoxide measured at 22°C using the superoxide dismutase (SOD)-inhibitable reduction of cytochrome *c* as previously described [28].

3. Results

3.1. mRNA expression of NADPH oxidase components by human fibroblasts

We have examined the mRNA expression of gp91-*phox*, p22-*phox*, p47-*phox* and p67-*phox* by cultured human fibroblasts using specific oligonucleotide primers (Table 1), designed from the known neutrophil cDNA sequences.

Total RNA was isolated from sub-confluent human fibroblasts, which had been cultured in 10% FCS. This RNA was reverse transcribed into cDNA and used as a template for RT-PCR. Amplification of this cDNA showed that fibroblasts express mRNA for p47-*phox*, p67-*phox* and p22-*phox*, at least within the designated primer regions (Fig. 1). The data obtained from RT-PCR suggested that the relative abundance of the p22-*phox* transcript was greater than that of p47-*phox* and p67-*phox*.

Oligonucleotide primers were designed corresponding to both the 5′ (gp915-*phox*: site of amplification 25–408 base pairs)

Table 1
Sequence information regarding the oligonucleotide primers used in this study

Gene	Primer sequence (5′-3′)	Product
actin*[1]	Forward GGAGCAATGATCTTGATCTT (20mer) Reverse TCCTGAGGTACGGGTCCTTCC (21mer)	204 bp
p22-phox	Forward GTTTGTGTGCCTGCTGGAGT (20mer) Reverse TGGGCGGCTGCTTGATGGT (19mer)	316 bp
gp915-phox	Forward TGGGCTGTGAATGAGGGGCT (20mer) Reverse TGACTCGGGCATTCACACAC (20mer)	383 bp
gp913-phox	Forward GCTGTTCAATGCTTGTGGCT (20mer) Reverse TCTCCTCATCATGGTGCACA (20mer)	403 bp
p47-phox	Forward ACCCAGCCAGCACTATGTGT (20mer) Reverse AGTAGCCTGTGACGTCGTCT (20mer)	767 bp
p67-phox	Forward CGAGGGAACCAGCTGATAGA (20mer) Reverse CATGGGAACACTGAGCTTCA (20mer)	746 bp

*[1]. Oligonucleotide primer sequences obtained from O'Bryan et al. (1991).

and 3′ (gp913-*phox*: site of amplification 1107–1510 base pairs) ends of the gp91-*phox* encoding transcript (Table 1). The results obtained using the gp913-*phox* pair of oligonucleotide primers are shown in Fig. 1. The RT-PCR specific amplification of α-actin (204 base pairs) was used as an internal standard. No expression of gp91-*phox* transcript could be detected in human fibroblasts (Fig. 1) using either set of primers even after

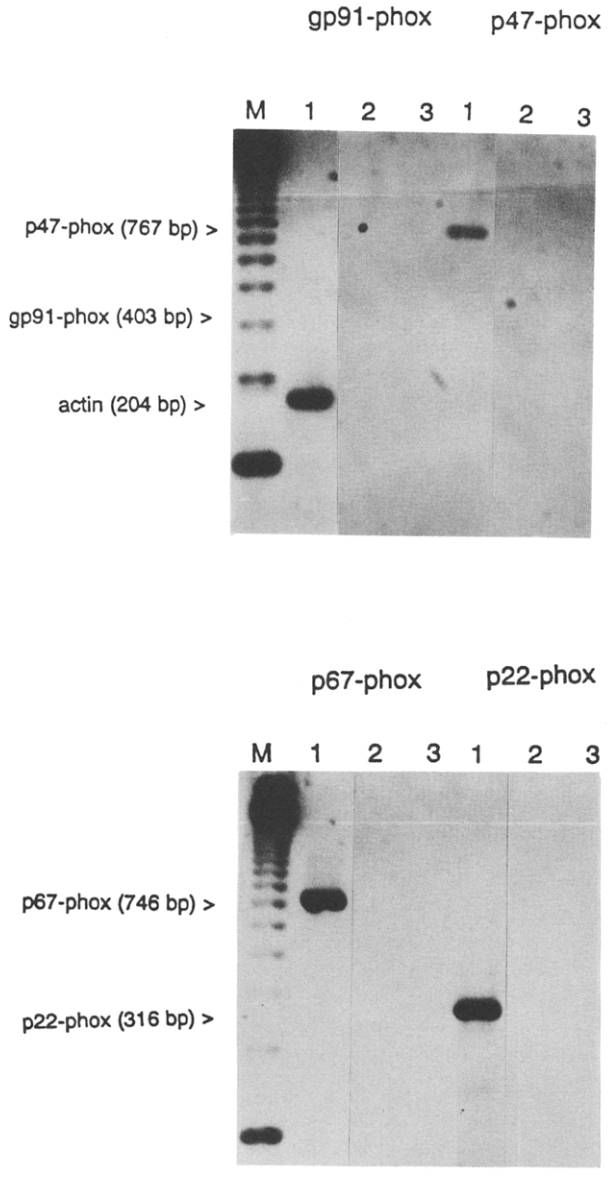

Fig. 1. RT-PCR amplification of NADPH oxidase components from human fibroblast cDNA. Total cellular RNA was isolated from human fibroblasts. 1 μg of total RNA was reverse transcribed into cDNA and used as a template in RT-PCR. Specific oligonucleotide primers were used to generate PCR products for p22-*phox* (25 cycles), gp91*phox* (gp913*phox*, 35 cycles), p47-*phox* (35 cycles) and p67-*phox* (38 cycles). A portion of gp91*phox* PCR amplification products was mixed with a portion from a 25 cycle α-actin (204 base pairs) amplification and separated on a 2% (w/v) NuSeive (Flowgen), 1% (w/v) agarose gel containing ethidium bromide. The separated products were visualised under UV illumination and sized using 123 base pair markers (Gibco-BRL) as molecular weight standards (M). Lane 1, human fibroblast cDNA; lane 2, reverse transcriptase-negative control reaction; lane 3, PCR-negative control reaction. gp913-*phox*, primer pairs located at the 3′ end of the gp91-*phox* transcript (target site, 1107–1510 base pairs).

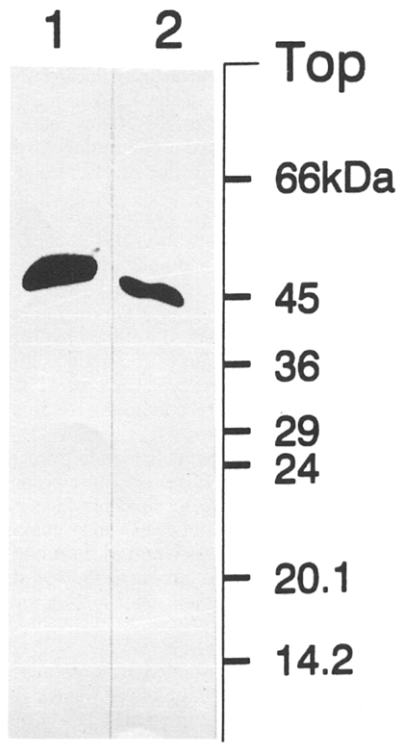

Fig. 2. Western blot analysis of human fibroblast cytosol using an anti-p47-*phox* antibody. Cytosolic proteins were isolated from human fibroblasts and concentrated by TCA-precipitation. The proteins were separated by SDS-PAGE on a 10% polyacrylamide gel and Western blotted. The proteins were probed with a polyclonal anti p47-*phox* antibody raised in rabbits against a C-terminal peptide (amino acid residues 367–390). Lane 1 represents 10 μg of PMA-activated neutrophil membranes, lane 2 represents 50 μg of human fibroblast cytosol.

40 amplification cycles (data not shown), indicating either a variation within, or absence of, this protein component.

3.2. *Protein expression of p47-phox*

To substantiate the expression of p47-*phox* by human fibroblasts, Western blot analysis was performed on fibroblast cytosolic proteins which had been concentrated by TCA-precipitation. This cytosolic fraction was probed with a polyclonal anti-p47-*phox* antibody (JW-1), which was raised against a C-terminal peptide of p47-*phox*. Immunodetection analysis showed the presence of a 47 kDa band in neutrophil cytosol, the electrophoretic mobility of which corresponds to a protein identified in human fibroblast cytosol (Fig. 2).

3.3. *Cell-free reconstitution*

To establish whether human fibroblast cytosol could maintain a SOD-inhibitable production of superoxide, a cell-free reconstitution assay was employed. Membrane and cytosol fractions obtained from fibroblasts and resting neutrophils were recombined (see Table 2) in the presence of GTPγ-S and sodium arachidonate. Superoxide generation was initiated by the addition of NADPH.

Table 2 shows that superoxide generation by either neutrophil of fibroblast membranes could be initiated by fibroblast cytosol. As in whole cells [14], rates of superoxide production in the fibroblast reconstitution system were low, approximately 1% of that of the neutrophil control.

4. Discussion

Meier et al. [17] previously reported the identification of a low potential cytochrome *b* ($E_{m,7} = -243$ mV) in the plasma membrane of human fibroblasts. This *b*-type cytochrome had spectroscopic characteristics similar to those of cytochrome b_{558}. Therefore a system analogous to the NADPH oxidase of phagocytic leukocytes may be a candidate for the production of fibroblast-derived superoxide.

The data presented in this manuscript demonstrates that human fibroblasts express mRNA for p22-*phox*, p47-*phox* and p67-*phox*. The relative abundance of the p47-*phox* and p67-*phox* transcripts were found to be lower than the expression of p22-*phox*. In addition a notable absence of detectable gp91-*phox* transcript was observed. A recent study by Meier et al. [20] showed that antibodies raised against the subunits of cytochrome b_{558} (gp91-*phox* and p22-*phox*) did not cross-react with membrane fractions isolated from human fibroblasts. In addition, they showed that fibroblasts isolated from a patient suffering from the X-linked form of chronic granulomatous disease (CGD), a genetic disorder which renders phagocytes unable to generate superoxide due to a defect within cytochrome b_{558}, were capable of generating an amount of superoxide equivalent to that produced by fibroblasts obtained from a healthy donor. These combined observations suggest that human fibroblasts may express an isoenzyme of NADPH oxidase. The apparent presence of an alternative *b*-type cytochrome capable of fulfilling the role of gp91-*phox* may be supported by the recent identification of a novel 30 kDa cytochrome *b* which shares a sub-cellular location with cytochrome b_{558} in activated and resting neutrophils [29].

Our RT-PCR data clearly demonstrate that human fibroblasts express mRNA for p22-*phox*. Expression of p22-*phox* has previously been reported in other non-phagocytic cell types [30]. However, Meier et al. [20] reported a lack of immunodetectable p22-*phox* in human fibroblasts. It has been suggested that the stability of cytochrome b_{558} is dependent upon the presence of both subunits (p22-*phox* and gp91-*phox*) as mutations within either subunit, as observed in X-linked forms of CGD, result in either trace amounts or a complete absence of the other subunit [13]. A similar situation has been observed using the baculovirus expression of cytochrome b_{558}, where expression of a stable cytochrome *b* was dependent upon the co-transfection of p22-*phox* and gp91-*phox* cDNA [31].

Table 2
Cell-free recostruction of human neutrophil and human fibroblast cell fractions. Cell-free reconstruction was performed as described in the Materials and Methods and the data obtained from two independent assays are shown. The production of superoxide is represented as nanomoles of superoxide/minute/μg of membrane protein

	nmoles sup./min/μg mem. protein	
	Exp. 1	Exp. 2
(a) Homologous cell free reconstitution		
5 μg Neutrophil membranes + 100 μg Neutrophil cytosol	15.60	16.10
5 μg Fibroblast membranes + 100 μg Fibroblast cytosol	0.16	0.18
(b) Heterologous cell free reconstitution		
5 μg Neutrophil membranes + 100 μg Fibroblast cytosol	0.20	0.19
5 μg Fibroblast membranes + 100 μg Neutrophil cytosol	0.10	0.13
(c) Control assays		
5 μg Neutrophil membranes only	0.00	0.00
5 μg Fibroblast membranes only	0.00	0.00
100 μg Neutrophil membranes only	0.00	0.00
100 μg Fibroblast cytosol only	0.00	0.00

RT-PCR showed that human fibroblasts, cultured in the presence of 10% FCS, express low levels of p47-*phox* and p67-*phox* mRNA. To confirm the presence of these cytosolic NADPH oxidase components we have used Western blot analysis. This identified a specific 47 kDa protein band in TCA-precipitated fibroblast cytosol. Previous reports have shown the protein expression of p47-*phox* in the hepatoma cell line HepG2 [26] and human glomerular mesangial cells (Jones et al., in press). The data obtained from similar experiments using an anti-p67-*phox* antibody were less conclusive and only suggested the presence of p67-*phox* in human fibroblasts, as additional protein bands were also identified (data not shown).

Cell-free reconstitution assays are widely used in the study of superoxide generation by phagocytes. Isolation of membranes and recombination with either cytosol or recombinant cytosolic proteins has allowed the identification of much of the protein components required for superoxide generation by these cells [32,11]. Using this assay sytem, it has previously been shown that the limited production of superoxide by EBV-transformed B-lymphocytes was due to a low amount of membrane-bound components as the levels of cytosolic factors approached those found in neutrophils [33]. We have found that cell-free reconstitution of fibroblast cytosol and membranes promoted a low level production of superoxide (approximately 1% of that generated by the neutrophil membrane-cytosol control). To establish whether fibroblast cytosol could mediate superoxide production by neutrophil membranes, fibroblast cytosol was mixed with resting neutrophil membranes. The generation of superoxide in this mixed cell reconstitution assay was also low, approximately 1–2% of the neutrophil control. This low level production of superoxide may be attributed to the relatively low abundance of fibroblast-derived cytosolic factors which may be rate-limiting for enzyme activity. Alternatively, the limited production of superoxide observed within this mixed-cell assay may relate to an incompatibility of the fibroblast components with those possessed by the neutrophil. These data, however, show that the enzymic activity of neutrophil membranes can be restored by fibroblast cytosol and therefore fibroblasts may contain factors related to the cytosolic components of the neutrophil NADPH oxidase.

Previous studies have demonstrated that the neutrophil p47-*phox* and p67-*phox* share a substantial degree of homology with the SH3 domains of non-receptor tyrosine kinases [9,10]. These domains may regulate the association of proteins to the cytoskeleton [34]. It has been proposed that the docking of p47-*phox* and p67-*phox* involves an interaction of SH3 domains with a proline-rich region of p22-*phox* [35]. It is therefore conceivable that the p47-*phox* and p67-*phox* homologues identified in human fibroblasts associate with the cytoskeleton in a similar fashion and this could support the presence of a p22-*phox* homologue in human fibroblasts.

In conclusion, the superoxide-generating system of human fibroblasts appears to be related to the NADPH oxidase of phagocytic leukocytes. A variation within the gp91-*phox* cytochrome *b* component may suggest the presence of an NADPH

oxidase isoenzyme, which as yet remains only partially characterised. The importance of this putative isoform may be significant since the presence of radical-generating isoenzymes have been documented for nitric oxide synthase [36,37] and haem oxygenase [38,39]. In particular, the presence of constitutive and inducible forms of nitric oxide synthase appear to correlate with enzymatic function (see [40] for review). Further studies are therefore required to examine the regulation of the fibroblast NADPH oxidase system, particularly in response to pro-inflammatory cytokines (IL-1β and TNFα), which have been shown to stimulate fibroblast production of superoxide [14,41]. Previous studies have reported that reactive oxygen species can induce fibroblast proliferation [42] and chemotaxis [43]. The production of reactive oxygen species may therefore be of importance in the inflammatory response, particularly in the development of fibrosis as observed in many chronic inflammatory diseases, such as idiopathic pulmonary fibrosis.

Acknowledgements: The authors would like to thank Dr. V.B. O'Donnell for providing the original cell cultures and Mr. C. Turner, Miss E.J. Hughes and Mr. A. Toye for technical assitance. Also thanks to Dr. A. Neubauer for designing PCR primers and Dr. B. Moser for critically reviewing this manuscript. This work was financially supported by grants obtained from the NKRF, KRUF, foundation of nephrology, the MRC and two studentships from the SERC.

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