The Expression of NADPH Oxidase Components in Human Glomerular Mesangial Cells: Detection of Protein and mRNA for p47phox, p67phox, and p22phox

Simon Arnott Jones, John Travers Hancock, Owen Tudor Grice Jones, Andreas Neubauer, and Nicholas Topley

S.A. Jones, Theodor Kocher Institute, Universitat Bern, Bern, Switzerland
J.T. Hancock, Department of Biological Sciences, University of the West of England, Bristol, United Kingdom
O.T.G. Jones, Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol, United Kingdom
A. Neubauer, Department of Haematology and Oncology, Universitaet Klinikum Rudolf Virchow, Charlo-

ABSTRACT

Previous work has shown that human mesangial cells (HMC) are capable of low rates of generation of reactive oxygen species for considerable periods of time. In this communication, the presence of components of an NADPH oxidase-like system, more commonly associated with phagocytic leukocytes, is shown. The ability of HMC to generate low levels of superoxide may have important implications in cellular signaling in general and may contribute to glomerular injury. Spectroscopic analysis of HMC membranes revealed a low-potential cytochrome b component, redox midpoint potential centered around -250 mV, which is present at 60 pmol/mg of membrane protein. Immunodetection studies suggested the presence of the p22phox, p47phox, and p67phox components of the NADPH oxidase, whereas the gp91phox was not detected. Further studies with oligonucleotide polymerase chain reaction primers showed that, in HMC the mRNA expression of the p67phox and p47phox was absent from growth-arrested cells but was present in HMC treated with interleukin-1β (1,000 pg/mL), whereas gp91phox could not be detected. Only mRNA corresponding to p22phox was present in growth-arrested cells; p47phox mRNA was induced by 2-h treatment with interleukin-1β but declined after 6-h treatment. These data illustrate for the first time that HMC are capable of expressing mRNA for several NADPH oxidase components. The apparent absence, or variation, of the gp91phox indicates the likelihood of an NADPH oxidase isoenzyme.

Key Words: Human mesangial cells, NADPH oxidase, superoxide, p47phox, p67phox, p22phox, gp91phox

In this communication, we describe an investigation of the superoxide-generating system of human kidney glomerular mesangial cells. These produce superoxide at a low rate in response to tumor necrosis factor-α (TNF-α), interleukin-1α (IL-1α) or IL-1β, and calcium ionophores (1,2). We have probed for the presence of mRNA for p22phox, gp91phox, p47phox, and p67phox, known components of NADPH oxidase of phagocytes. Expression of each of these mRNA was undetectable in growth-arrested cells, but some components were expressed after IL-1β treatment and we describe their characterization.

When phagocytic leukocytes recognize and bind to infecting microorganisms, they release superoxide into the developing phagosome. Superoxide, and products derived from it, are cytotoxic and contribute to the killing of the infecting organism. The enzyme complex that forms superoxide, NADPH oxidase, is believed to contain several components. In the plasma membrane of neutrophils, there is a low-potential cytochrome b with two subunits (p22phox and gp91phox) (3,4), which in addition to its prototetide prosthetic group, has some regions of homology with known FAD-binding proteins (5); in the cytosol, three protein components of the NADPH oxidase are found (called p47phox, p67phox, and GTP-binding protein, p21rac (6)). These translocate from the cytosol to the plasma membrane and associate with the cytochrome b when the neutrophil is stimulated. A very simplified representation of the oxidase is given in Figure 1.

Superoxide is produced by a variety of nonphagocytic cells (see review by Cross and Jones [7]), although its function is unclear and the nature of the oxidase(s) involved in many cases remains to be established. The most active of these systems has been found in Epstein-Barr virus-transformed B lympho-
cytes (8-10), which generate superoxide at 1 to 5% of the rate found in phagocytes and which contain the low-potential cytochrome b and the cytosolic factors characteristic of the neutrophil oxidase. In peripheral blood or tonsillar nontransformed B lymphocytes, superoxide was generated in response to the cross-linking of surface immunoglobulin (11) and was associated with the expression of cytochrome b_245 (11,12). This superoxide production may be involved in some form of immunoregulation. Fibroblasts secrete superoxide in response to cytokine treatment, and it has been observed that superoxide promotes their proliferation (13-16), suggesting that it has some signaling function.

We believe our report is the first to provide evidence that mRNA for p22phox, p47phox, and p67phox are expressed in cells derived from nonhemopoetic tissue. It suggests that NADPH oxidase may be widespread in mammalian cell types and, like nitric oxide synthase, has roles in signaling as well as combating infection. The superoxide generated may contribute to glomerular injury.

**MATERIALS AND METHODS**

**Isolation and Culture of Human Mesangial Cells**

Mesangial cells were isolated from normal human kidney sections obtained from renal carcinoma patients undergoing nephrectomy by the method of Lovett et al. (17). Mesangial cells were grown in RPMI-1640 or minimal essential medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM l-glutamine, 5 μg/mL bovine insulin, 5 μg/mL human transferrin, and 10% (vol/vol) fetal calf serum (FCS). When the cells reached confluency, they were washed in sterile phosphate-buffered saline (PBS) and trypsinized with a 1:1 mixture of 0.25% (wt/vol) trypsin and 0.02% (wt/vol) EDTA, both containing 0.1% (wt/vol) glucose. The trypsin was neutralized with 10% (vol/vol) FCS containing endogenous trypsin inhibitor, and the cells were subcultured. The mesangial cells were subsequently characterized by immunocytochemistry, as described by Radeke et al. (1).

For the transfer of cells to serum-free medium, they were grown to subconfluence in culture medium containing 10% (vol/vol) FCS. The cells were then washed with several changes of sterile PBS, and serum-free MCDM-302 medium (Sigma Chemical Co., Poole, United Kingdom) was added, containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM l-glutamine, 5 μg/mL bovine insulin, and 5 μg/mL human transferrin. After 48 h, the cells were considered growth arrested (tissue culture G0), as described by Plöge et al. (18).

**Determination of Superoxide Production**

Cells harvested by trypsinization were counted, seeded at a fixed density, and allowed to adhere overnight in 24-well microtiter plates at 37°C, 5% CO₂. The cells were washed with several changes of Krebs-Ringer buffer, and 800 μL of Krebs-Ringer buffer containing 80 μM cytochrome c, plus or minus stimuli, was added to each well. The mixture was incubated for 2 h at 37°C, 5% CO₂. Cytochrome c reduction was determined spectrophotometrically at 550 nm. Control samples were incubated in the presence of 0.16 mg/mL superoxide dismutase. The production of superoxide was determined with the extinction coefficient of cytochrome c at 550 nm = 19.1 mM/cm. Cell numbers were obtained by trypsinizing four randomly selected wells after the assay and counting. The following rates were recorded: unstimulated cells, 4.42 ± 0.40; TNF (10 ng/mL)-stimulated cells, 6.00 ± 0.31; ionomycin (5 μM)-stimulated cells, 15.90 ± 1.40. Rates of superoxide production by growth-arrested cells were too low for accurate measurement, with ionomycin as stimulus.

**Preparation of Membranes and Cytosol**

Human mesangial cells (HMC) were grown until fully confluent. The medium was discarded, and the cells were placed on ice for 10 to 15 min in 5 to 10 mL of extraction buffer containing 50 mM Tris–1 mM EDTA (pH 7.4), supplemented with 1 mM phenylmethylsulfonyl fluoride and DFP. This buffer was removed, and the flasks were broken open. Extraction buffer (400 μL) was added, and the cells were scraped into one corner and collected by aspiration. The suspension was sonicated on ice with three 15- to 30-s bursts at 50 W. Nuclear and mitochondrial contamination was removed by centrifugation (1,200g for 15 min at 4°C). The supernatant was recentrifuged (100,000g for 45 min at 4°C), and the supernatant containing the cytosolic proteins...
Arlington Heights, IL) for 1 h (19). Determined was 25 mM Ths, 192 mM glycine, and 20% wash with 5% (wt/vol) dried milk powder and containing the Society of Nephrology Journal of the American Cross was collected. The pelleted membranes were resuspended in 15 mL) suspended in PBS baseline oxidized spectrum was recorded. A few grains of sodium dithionite were added to the sample cuvette, and the reduced spectrum was recorded. The reduced-minus-oxidized spectrum was then plotted. For CO-difference spectra, a baseline was recorded after the addition of sodium dithionite to both reference and sample cuvettes, and then, CO was bubbled gently into the sample cuvette for 1 min before the CO-difference spectrum was recorded. For the potentiometric difference, spectroscopy samples placed in a cuvette fitted with a platinum electrode were flushed with O2-free argon and spectra were recorded at different, known, oxidation-reduction potentials.

Electrophoresis and Immunoblotting

Protein fractions (10 to 15 μg/track) separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were electroblotted onto Hybond-ECL (Amersham, Arlington Heights, IL) for 1 h at 100 V on ice. Transfer buffer was 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol. The sample membrane was blocked overnight in PBS containing 5% (wt/vol) dried milk powder and 0.25% Tween 20. The membrane was washed in buffer containing 150 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA, and 0.2% Tween 20 (pH 7.5) and agitated for 90 min in wash buffer supplemented with 5% (wt/vol) dried milk powder and containing the primary antibody. The membrane was washed, and the secondary antibody was added for an additional 90 min in wash buffer containing 5% (wt/vol) dried milk powder. The blot was washed and developed by use of the ECL western blotting detection system (Amersham).

Preparation of Total RNA

Total RNA was extracted from HMC with RNAzol B (Biogenesis Ltd., Bournemouth, United Kingdom). Vacuum-dried RNA was resuspended in diethylpyrocarbonate-treated water and precipitated at −70°C with 0.5 vol of 8 M ammonium acetate and 2.5 vol of absolute ethanol. RNA concentrations were determined spectrophotometrically.

The integrity of the RNA was determined by assessment of the 260 nm:280 nm ratio, by electrophoresis of the samples on a 2% (wt/vol) agarose gel, containing 25 μg of ethidium bromide, with a Tris acetate EDTA (TAE) electrode buffer (0.8 M Tris–5 mM EDTA–2.28% vol/vol acetic acid), and by visualization by UV transillumination.

Preparation of cDNA and Polymerase Chain Reaction

These procedures were performed as described by Topley et al. (21).

Sequencing of Polymerase Chain Reaction Products

Reamplified polymerase chain reaction (PCR) products were gel purified and ligated into the Smal, bovine alkaline phosphatase-treated pUC 18 (Pharmacia, Piscataway, NJ), which was subsequently transformed into SURE cells (Promega, Madison, WI) a recA− strain of Escherichia coli. Transformed plasmids containing the appropriate insert were selected and sequenced.

Isolation of Blood Monocytes

Buffy coats (obtained from Southmead Regional Blood Transfusion Centre, Bristol) were mixed with an equal volume of 2% (wt/vol) dextran in PBS containing 0.8% (wt/vol) trisodium citrate. After standing for 20 min at an angle of 45 degrees, the straw-colored upper layer was aspirated off, underlayered with lymphoprep (Nycomed Pharma AS, Oslo, Norway), and centrifuged at 800g for 20 min. The resulting interface was collected. The monocytes were harvested by centrifugation (400g for 10 min) and washed repeatedly with PBS containing 0.4% (wt/vol) trisodium citrate to reduce lymphocyte and platelet contamination. The cells were counted and allowed to adhere to six-well microtiter plates for 2 h at 37°C, 5% CO2, at a cell density of 105 cells/well. The nonadherent cells were removed by washing, and the cells were cultured in RPMI-1640 medium supplemented with 10% human serum (obtained from Southmead Regional Blood Transfusion Centre), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine.

RESULTS

Previous work (1,2) has shown that HMC secrete superoxide into the medium in response to a number of signals. We confirmed these results for our cell isolates. A constant rate of superoxide release was seen in HMC, even in the absence of a stimulus, which is usually restricted to a burst lasting approximately 5 min.

Absorption spectra show that plasma membranes from HMC appear to contain a cytochrome of the b type (2) with absorption maxima at 558, 530, and 428 nm (Figure 2). This has a low oxidation-reduction midpoint potential (E0m) more negative than −162 mV (Figure 2) and binds CO with low affinity (Figure 2). This may be implicated in oxidase activity. Mitochondrial cytochromes have Em above 100 mV and so would be fully reduced by −162 mV. The component not reduced until more negative potentials are applied (e.g., −310 mV) is not part of the respiratory chain but
Absorbance

A. 1 2 3

Wavelength (nm)

B. TOP

Figure 2. Difference spectra of HMC membrane fractions. Sodium dithionite-reduced minus air-oxidized difference spectra (top) of HMC membranes (1.1 mg of protein/mL in PBS). Reduced membranes were saturated with carbon monoxide, spectra were recorded, and reduced spectrum was subtracted (bottom). The insert shows the low-potential component (reduced at -310 mV minus reduced at -162 mV); membrane concentration, 1.8 mg of protein/mL.

Figure 3. Immunodetection of NADPH oxidase component HMC fractions. (A) Western blot analysis for gp91phox. Proteins were separated on a 8% polyacrylamide gel. Lane 1, 10 μg of phorbol myristate acetate (PMA)-activated neutrophil membranes; Lane 2, 10 μg of PMA-activated neutrophil cytosol; Lane 3, 15 μg of HMC membranes. (B) Immunoprecipitation of p47phox. Proteins were separated on a 10% polyacrylamide gel. Lane 1, SDS, seven molecular weight standards; Lane 2, antibody/PBS control. Lane 3, Immunoprecipitate from 150 μg of HMC cytosol.

resembles cytochrome b$_{245}$ of neutrophil NADPH oxidase. This is supported by the detection of the low level of CO binding, also typical of cytochrome b$_{245}$. We have attempted to determine whether other components similar to those necessary for the neutrophil NADPH oxidase may also be present. Antibodies to p22phox, p47phox, and p67phox gave unclear results when used in immunodetection experiments with HMC cytosol and membranes. We could not detect any band at the appropriate molecular mass staining with antibody to gp91phox (Figure 3A). Other proteins that were labeled with the polyclonal antibody may bear common epitopes, e.g., nucleotide-binding sites. Antibodies to p47phox and p67phox gave bands at the expected molecular mass, but they too were accompanied by additional labeled bands of different molecular masses. Western blotting analysis with a polyclonal antibody raised against the p22phox gave a single 22-kd band in neutrophil membranes. In HMC membrane fractions, a 22-kd band was also labeled; however, a number of unidentified higher molecular weight bands were also detected and the result was ambiguous. Probing HMC cytosol and red blood cell membranes with this antibody failed to detect a protein with a molecular mass of 22 kd. Evidence for other NADPH oxidase components was sought. Immunoprecipitation of HMC cytosol with antibody to p47phox yielded a complex that separated on SDS-PAGE and clearly showed a band at 47 kd, together with the band of the antibody heavy chain (Figure 3B). However the polyclonal antibody to p67phox labeled most strongly a protein of 47 kd together with the expected protein of 67 kd (results not shown).
Detection of mRNA for p22phox, p47phox, and p67phox in HMC

The immunologic testing described above clearly indicated that p47phox was expressed in HMC and suggested that p22phox and p67phox were also expressed in low amounts. We therefore designed and synthesized oligonucleotide primers for use in PCR experiments to test the expression of the corresponding mRNA (Table 1). RNA fractions were extracted and purified from both growth-arrested and IL-1β-treated cells, and cDNA was prepared from them by standard techniques. The cDNA was used as a template for the PCR. RNA samples prepared from monocytes that were known to express p22phox, gp91phox, p47phox, and p67phox were run for comparison, to ensure that the primers were effective. When the PCR products were analyzed by agarose gel electrophoresis, bands corresponding to fragments of the predicted base pair size were seen in the monocyte-derived products (Figure 4). Clear bands, corresponding to p22phox, p47phox, and p67phox PCR products, were seen in the HMC tracks derived from IL-1β-treated cells. Bands corresponding to p22phox and p67phox were absent from growth-arrested HMC, but p22phox was constantly expressed (Figure 5). The expression of p22phox was always higher than that of p47phox and of p67phox, which was the lowest. There was no evidence for the presence of a product corresponding to gp91phox, even after 40 cycles of PCR amplification in either growth-arrested or IL-1β-treated mRNA fractions. PCR products of α-actin mRNA were used as an internal standard to illustrate equal loading of cDNA samples (Figure 5).

DNA bands extracted from the agarose gel and reamplified by PCR, with the appropriate primers, gave strong bands of the expected size when analyzed.

![Figure 4. PCR-amplified NADPH oxidase components from human monocytes.](image)

These products were extracted from the gel, ligated into a pUC18 plasmid vector, and used to transform a recA" strain of E. coli. After growth overnight, the plasmid DNA containing the appropriate insert was isolated and sequenced with M13 universal primers. Within the region selected, the sequences obtained were more than 98% identical with the corresponding sequences of human neutrophil p22phox, p47phox, and p67phox cDNA.

Additional Evidence for the Lack of mRNA for gp91phox in HMC

Primers corresponding to putative nucleotide regions of gp91phox were designed and used in PCR.

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<th>Table 1. Information on the sequences of oligonucleotide primers designed from the known neutrophil cDNA sequences</th>
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\(^{a}\) Reference 41, O'Bryan et al. (1991).
\(^{c}\) Reference 43, Royer-Pokora et al. (1986). Accession No. X04011.
Regulation of Expression of mRNA of p47phox and p67phox

When HMC were transferred to serum-free medium, the content of mRNA for p47phox declined to levels undetectable after 48 h. At the same time, the capacity to form superoxide declined to undetectable levels (see Materials and Methods). In contrast, p22phox and α-actin were unaffected by this treatment, confirming the view that p22phox mRNA is constitutive. After a 2-h exposure to IL-1β, p47phox mRNA was greatly enhanced, but after 6 h, it had greatly declined (Figure 7).

DISCUSSION

Superoxide is produced by a wide variety of cell types (7), and it is not clear whether the mechanism for its formation is common to all, or many, cell types and whether it has a biological function when secreted at low rates. This study shows for the first time that many of the components of phagocyte NADPH oxidase are present in human glomerular mesangial cells. Neutrophils produce superoxide at high rates (around 3 to 5 nmol/min per 10^-6 cells) during a respiratory burst activated by receptor interaction with opsonized bacteria or by a variety of soluble stimuli. The NADPH oxidase complex involved is an association of cytosolic and membrane proteins that are now well characterized. Superoxide production by neutrophils is clearly linked to the microbicidal functions of these cells.

Our results clearly show for the first time that mRNA for p47phox and p67phox components of the superoxide-generating oxidase system are present in HMC stimulated with IL-1β, while confirming the constant presence of mRNA for p22phox. It is probable...
The expression of the oxidase components in growth-arrested cells followed the addition of IL-1β. It has been reported that IL-1α/β and TNF-α act as factors that modulate the proliferation of HMC (25,26) in culture. These factors also lead to superoxide formation by HMC. It is possible that reactive oxygen species participate in the maintenance of the proliferative phase. It is interesting that growth-arrested mesangial cells do not express RNA for p47 phox or p67 phox. These oxidase components are synthesized only in response to proinflammatory mediators.

The potential of superoxide to cause tissue damage is well known (7), but the pathophysiologic effect of HMC-produced superoxide is uncertain. The local production of free radicals might contribute to the remodeling of the glomerulus, characteristic of renal disease. Free radicals have been shown to modulate basement membrane breakdown (30), increase cell injury, and modulate cell proliferation (13), all features of glomerular scarring.

In acute experimental glomerulonephritis, evidence suggests that the infiltration of macrophages and neutrophils contributes to the development of glomerular injury (31). However, the absence of phagocytic leukocytes in other forms of nephritis, for example, membrane proliferative glomerulonephritis, indicates the findings of Radeke et al. (2), who indicated the presence of a membrane-bound HMC cytochrome b analogous to that found in neutrophils. Although our results confirm the presence of a low-potential cytochrome b in HMC membranes, both protein and mRNA of the gp91 phox were lacking. It is possible that the antibody epitope reported by Radeke et al. (2) is within a conserved region of the cytochrome b, common to both mesenchymal and leukocyte cell types. Our results suggest that the anti-gp91 phox antibody used by us recognizes a unique epitope within the neutrophil variant that is absent in HMC. It appears possible that an isoenzyme of cytochrome b-245 is present in these cells, with an altered gp91 phox subunit. Such a possibility has been suggested for fibroblasts, where superoxide-generating activity was retained in cells from a chronic granulomatous disease patient whose neutrophils lacked the b-type cytochrome (16).

It is possible that low-level generation of superoxide or molecules formed from it, such as H2O2, could also act as signal molecules as well as cytotoxic agents. In T cell lines, reactive oxygen species at low doses (around 30 μM) activate the transcription factor NF-κB by causing the release of the inhibitory subunit IκB (23). H2O2 can stimulate guanylate cyclase and so affect vascular tone (24). H2O2 also promotes tyrosine phosphorylation (25), perhaps through the activity of an oxidant-sensitive kinase. Low levels of oxidants cause a rapid rise in the specific activity of soluble protein kinase C (26). If activated oxygen acts as a second messenger, as well as a cytotoxic agent, it would resemble nitric oxide and, perhaps, carbon monoxide (27) in this joint function.

Figure 7. Time-dependent changes in expression of mRNA for p47 phox and p22 phox in mesangial cells and induction by IL-1β. Total RNA was isolated from HMC cultures that had been cultured under various conditions and reverse transcribed into cDNA. A portion of the p47 phox (35 cycles) PCR was mixed with portions of the p22 phox and α-actin (both 25 cycles) amplifications. The complete mixture was separated on a 2% (wt/vol) NuSieve-1% agarose gel with ethidium bromide. The DNA products were visualized under UV illumination and sized with 123-base-pair markers (Gibco-BRL) as molecular weight standards. Lane 1, HMC cDNA isolated from FCS-cultured cells; Lane 2, HMC cDNA isolated from 24 h growth-arrested cells; Lane 3, HMC cDNA isolated from 48 h growth-arrested cells; Lane 4, HMC cDNA isolated from 48 h growth-arrested cells and cultured for another 2 h in 1,000 pg/ml IL-1β; Lane 5, HMC cDNA isolated from 48 h growth-arrested cells and cultured for another 6 h in 1,000 pg/ml IL-1β; Lane 6, negative-PCR control (no DNA).

that p22 phox, p47 phox, and p67 phox function as part of an NADPH oxidase activity in HMC. These proteins and their messengers are present at very low concentrations, even in IL-1β-induced cells, and may be rate limiting in the process of superoxide formation. The mRNA for p67 phox was always less abundant than that for p47 phox. It is interesting that the mRNA for p22 phox was the most abundant, as detected by PCR in mesangial cells. This mRNA has previously been reported to be present in a variety of cell types that did not apparently express NADPH oxidase, and its level was described as high in whole mouse kidney mRNA (22), although mRNA for gp91 phox was not detected. Our results agree with this, although we clearly see a low-potential cytochrome b in mesangial cell membranes (Figure 2).

The observations reported here appear to contradict...
that intrinsic glomerular cells are also important in the development of the inflammatory response within the glomerulus. Previous studies have shown that the addition of reactive oxygen species (ROS) to glomerular cells can regulate the production of several cell mediators, such as prostaglandins and thromboxanes (32–34). This may indicate a role for low-level superoxide production in cellular signaling. It is also possible that mesangial cell-derived ROS function in the activation/inactivation of particular proteins, such as proteases and protease inhibitors. The activation of metalloproteases by ROS has been shown to be involved in the degradation of the glomerular basement membrane, which leads to elevated levels of proteinuria. Certainly, rat mesangial cells cultured in macrophage-conditioned medium or IL-1 induce neutral protease activity (35), and in a recent study, the induction of pgp91phox by visceral epithelial cells correlated with enhanced proteinuria in passive Heymann nephritis (36). The in vitro data presented here suggest that the induction of a fully active NADPH oxidase requires a proliferative stimulus or activation by proinflammatory cytokines, because the expression of p47phox and p67phox was only detected in FCS or IL-1β-treated cells. It is known from animal studies of glomerular nephritis that reducing IL-1 (or TNF) synthesis results in a reduction in the severity of glomerular injury (37–40). These data suggest that IL-1 levels are elevated during disease; it is possible that mesangial cell NADPH oxidase activity might be induced (by cytokines) during inflammation and contribute to the progression of glomerular injury. The absence of reverse transcription-PCR-detectable transcripts for p47phox and p67phox in growth-arrested mesangial cells suggests that they are important in the regulation of this inducible enzyme complex and that their active expression may occur as a result of glomerular inflammation. Our results show clearly for the first time that the cytotoxic factors p47phox and p67phox are expressed in nonlymphoid cells and suggest that an NADPH oxidase system analogous to that found in leukocytes is of widespread distribution.

ACKNOWLEDGMENTS

We acknowledge the following: Dr. A. McVey, Department of Pathology, Southmead Hospital, Bristol, for supply of kidney tissue; Ravinder Chana, Institute of Renal Medicine, University of Cardiff, for supplying HMC isolates; Dr. J. Curtinette and Professor A.W. Segal for supplying antibodies; and Southmead Regional Blood Transfusion Centre for supply ofuffy coats. Helen Downer is thanked for DNA sequencing. This work was funded by grants from NKRF, KRUF, Foundation for Nephrology, the Wellcome, Trust and a studentship from the SERC. Mrs. Elaine Anderson is thanked for help in preparation of the manuscript.

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