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Short Sequence-Paper

Cytochrome *b*-558 α-subunit cloning and expression in rat aortic smooth muscle cells

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Abstract

Recent studies have shown that the NADPH oxidase participates in the generation of superoxide anion in non-phagocytic cells. Here we report the isolation and nucleotide sequence of a cDNA for the cytochrome *b*-558 α-subunit of the NADPH oxidase in rat vascular smooth muscle cells (VSMCs). The coding region of the cDNA was 93% homologous to mouse and 81% to human in nucleotide sequence and 96% homologous to mouse and 89% to human in the deduced amino acid sequence. Our results provide a tool with which to explore the mechanism of superoxide anion generation in rat VSMCs and other non-phagocytic cells.

Keywords: Vascular smooth muscle; Superoxide anion; NADPH oxidase; Cytochrome *b*-558; cDNA cloning

Superoxide anion formation has been mainly described as a specific function of phagocytes such as neutrophils, macrophages, and monocytes [1]. The generation of superoxide anion is catalyzed by the membrane-bound multicomponent NADPH oxidase system in phagocytes. This consists of two cytosolic 47 kDa and 67 kDa components (p47 phox and p67 phox) [2,3], a small-molecular-weight G-protein (rac-1 or rac-2) [4,5], and a membrane-associated cytochrome *b*-558. Cytochrome *b*-558 is a heme-carrying heterodimer with a non-glycosylated 22 kDa α-subunit (p22 phox) and a glycosylated 91 kDa β-subunit (gp91 phox), and functions as the final electron transporter from NADPH to oxygen in generating superoxide anion [6].

Recent studies have shown that the ability to generate superoxide anion is not limited to phagocytes. Superoxide anion generation has been demonstrated in non-phagocytic cells including B-lymphocytes [7], fibroblasts [8], glomerular mesangial cells [9], smooth muscle cells [10], endothelial cells [11], and epithelial cells [12]. Furthermore, oxygen free radicals such as superoxide anion have been implicated in the pathogenesis of both hypertension and atherosclerosis [13]. Despite the potential importance of superoxide anion, the pathways leading to its generation in vascular cells remain unclarified.

Recently, we reported that angiotensin II stimulates superoxide anion generation by activating both the NADH and NADPH oxidases in cultured rat vascular smooth muscle cells (VSMCs) [14]. Our data suggested that superoxide anion may participate in the signal transduction system related to cell growth such as hypertrophy or hyperplasia. We hypothesized that the NADPH oxidase system may be composed of some of the same components as that of phagocytes, and wished to determine the existence, expression and regulation of each of these components in VSMCs.

We first sought to determine whether the mRNA of the cytochrome *b*-558 α-subunit is expressed in rat VSMCs. VSMCs were isolated from 200–250 g male Sprague-Dawley rat thoracic aorta by enzymatic digestion as described previously [15] and total RNA was isolated with the acid-phenol extraction method [16].

Reverse transcriptional polymerase chain reaction (RT-PCR) was carried out using GeneAmp RNA PCR Kit (Perkin Elmer). Single-stranded cDNA was prepared from total RNA of rat VSMCs with an oligo(dT) primer and reverse transcriptase by incubating for 30 min at 42°C. The resulting single-stranded cDNA was amplified using synthetic oligonucleotide primers based on the cytochrome *b*-558 α-subunit of mouse macrophage [17]; upstream

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primer 5′-ATTCTCATCACTGGGGGCATCGTG-3′ (54–77) and downstream primer 5′-TCCTTGGGTTTAG-GCTCAATGGGAG-3′ (415–391). PCR amplification was performed with deoxynucleoside triphosphate and Taq DNA polymerase for 30 cycles; 1 min at 95°C, 2 min at 50°C and 1.5 min at 72°C. The PCR product migrated as a clear single band on gel electrophoresis and the molecular size was 360 bp, which is in accord with that expected from the mouse cDNA sequence [17].

After purification of the PCR product, Cycle Sequencing was performed in one of four dideoxynucleoside triphosphate-containing reaction mixtures and 50 fmol purified PCR product, 1.6 pmol of either sense or antisense [^{32}P]-5′ end-labeled primers identical to those used for

(A)

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-44                  CCACGCGTCCGGGACCGAGCGGCTGCGTGTGCTGGGTCCTCACC

  1 ATG GGG CAG ATC GAG TGG GCC ATG TGG GCC AAC GAA CAG GCG CTG
    M   G   Q   I   E   W   A   M   W   A   N   E   Q   A   L    15
 46 GCA TCT GGC CTG ATC CTC ATC ACA GGG GGC ATC GTG GCT ACT GCG
    A   S   G   L   I   L   I   T   G   G   I   V   A   T   A    30
 91 GGA CGC TTC ACG CAG TGG TAC TTT GGT GCT TAC TCT ATT GTT GCA
    G   R   F   T   Q   W   Y   F   G   A   Y   S   I   V   A    45
136 GGA GTG CTC ATC TGT CTG CTG GAG TAC CCC CGG GGA AAG AGG AAA
    G   V   L   I   C   L   L   E   Y   P   R   G   K   R   K    60
181 AAG GGC TCC ACC ATG GAG CGG TGT GGA CAG AAG TAC CTG ACC GCT
    K   G   S   T   M   E   R   C   G   Q   K   Y   L   T   A    75
226 GTG GTG AAG CTG TTC GGG CCC CTC ACC AGA AAT TAC TAC GTC CGG
    V   V   K   L   F   G   P   L   T   R   N   Y   Y   V   R    90
271 GCT GTC CTC CAC TTA CTG CTG TCC GTG CCT GCA GGC TTC CTG CTG
    A   V   L   H   L   L   L   S   V   P   A   G   F   L   L   105
316 GCC ACC ATC CTG GGG ACC GTC TGC TTG GCC ATT GCC AGT GTG ATC
    A   T   I   L   G   T   V   C   L   A   I   A   S   V   I   120
361 TAC CTG CTG GCA GCC ATC CGG GGT GAG CAG TGG ACT CCC ATT GAG
    Y   L   L   A   A   I   R   G   E   Q   W   T   P   I   E   135
406 CCT AAA CCC AAG GAG CGG CCG CAG GTT GGA GGC ACC ATC AAG CAG
    P   K   P   K   E   R   P   Q   V   G   G   T   I   K   Q   150
451 CCA CCT ACC AAC CCC CCA CCC CGG CCA CCA GCG GAG GTC CGC AAG
    P   P   T   N   P   P   P   R   P   P   A   E   V   R   K   165
496 AAG CCA AGT GAG GCC GAA GAG GAG GCA GCC TCG GCA GGA GGA CCC
    K   P   S   E   A   E   E   E   A   A   S   A   G   G   P   180
541 CAG GTT AAC CCA ATT CCA GTG ACA GAT GAG GTC GTG TGA
    Q   V   N   P   I   P   V   T   D   E   V   V              192

580 CCTTCAGTGGCTCCTGCTTGAGTTTCCAAGGTGTTGCTCCCAGAGGGTGGTGATGCCCAC
640 TGTAATAAACATGGTTAATAGCTGGTAAA
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(B)

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rat VSMCs          MGQIEWAMWANEQALASGLILITGGIVATAGRFTQWVFGAYSIVAGVLI
mouse macrophage   --**********************************************A***LI
human neutrophil   ***********************************************V***FV

                                    †                              ‡
rat VSMCs          CLLEYPRGKRKKGSTMERCGQKYLTAVVKLFGPLTRNYYVRAVLHLLLS
mouse macrophage   ******************C***YL*S*******L********A**F***
human neutrophil   ******************W***HM*A*******F********V**L***

                                                                   †
rat VSMCs          VPAGFLLATILGTVCLAIASVIYLLAAIRGEQWTPIEPKPKERPQVGGT
mouse macrophage   ************V******V******I************K****V***
human neutrophil   ************A******G******V************R****I***

rat VSMCs          IKQPPTNPPPRPPAEVRKKPSEAEEEAASAGGP•••QVNPIPVTDEVV
mouse macrophage   *****T*********V******G*EE**SA*G*•••••***M*******
human neutrophil   *****S*********A******E*AA**AG*P*GGP****I*******
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Fig. 1. (A) Nucleotide sequence of the cDNA and deduced amino-acid sequence of cytochrome *b*-558 α-subunit of rat VSMCs. The 5′ untranslated nucleotide sequence is negatively numbered from the first base upstream of the initiator codon. A potential polyadenylation signal in the 3′ untranslated region is underlined. (B) Alignment of the amino acid sequences of the cytochrome *b*-558 α-subunit of rat VSMCs, mouse macrophages [17], and human neutrophils [18]. * Amino acid residues identical among the three species. • Indicates the positions of amino acids which are found only in the human neutrophil. † Indicates potential phosphorylation sites by protein kinase C. ‡ Indicates the putative heme-carrying histidine residue.

PCR, 2 units of AmpliTaq DNA polymerase, 40 μM 7-deaza-dGTP, 20 μM dATP, 20 μM dTTP, and 20 μM dCTP (AmpliTaq Cycle Sequencing Kit, Perkin Elmer). The reaction conditions were as follows: 1 min at 95°C for 1 cycle and 1 min at 95°C, 2 min at 50°C, and 1.5 min at 72°C for 20 cycles. Primer 5′ end labeling was performed with 10 μCi [γ-^{32}P]ATP and 30 units of T4 polynucleotide kinase. The reaction products were loaded on 6% polyacrylamide gel electrophoresis and exposed to film at −80°C. Sequencing was performed on three separate preparations.

Direct sequencing showed that the PCR product exhibited very high similarity with the mouse cDNA sequence between the upstream and downstream primers. Northern blotting analysis of rat VSMCs using the PCR product as a probe resulted in detection of a single band of molecular size 0.8 kb. This size is in agreement with the size of cytochrome *b*-558 α-subunit mRNA in human [18] and mouse [17]. No other sequence in GenBank was similar to the PCR product, except for those of cytochrome *b*-558 α-subunits. Therefore, the PCR product was considered to be specific and was used to screen a cDNA library of rat VSMCs.

We used a size-fractionated cDNA library of rat VSMCs which is cloned at the *Not*I-*Sal*I sites of the pSPORT vector [19]. Approximately $5 \cdot 10^4$ independent clones were screened on membranes with the [^{32}P]dCTP-labeled PCR product fragments. Nine positive clones were obtained during the secondary screening. Double-stranded cDNA inserted in pSPORT was sequenced after alkaline denaturation by the dideoxynucleotide chain termination method [20] using Sequenase Version 2.0 (United States Biochemical). Only one clone coded the full length rat cytochrome *b*-558 α-subunit. The T7 and SP6 promoter primers (Promega) and four additional synthetic oligonucleotide primers from partial sequence data were used to obtain complete sequence in both directions.

Fig. 1A shows the nucleotide sequence and the deduced amino acid sequence of rat cytochrome *b*-558 α-subunit. The sequence of 709 nucleotides reveals a 579-nucleotide open reading frame extending from the start codon ATG to the termination codon TGA. A consensus polyadenylation signal AATAAA is present at position 643–648. This cDNA sequence encodes a 192-amino-acid protein with an estimated molecular weight of 20745. Comparison of the deduced amino acid sequence with those of the cytochrome *b*-558 α-subunit of human neutrophil and mouse macrophage is shown in Fig. 1B. The coding regions of the rat cytochrome *b*-558 α-subunit conserve 93% homology with mouse and 81% with human in the nucleotide

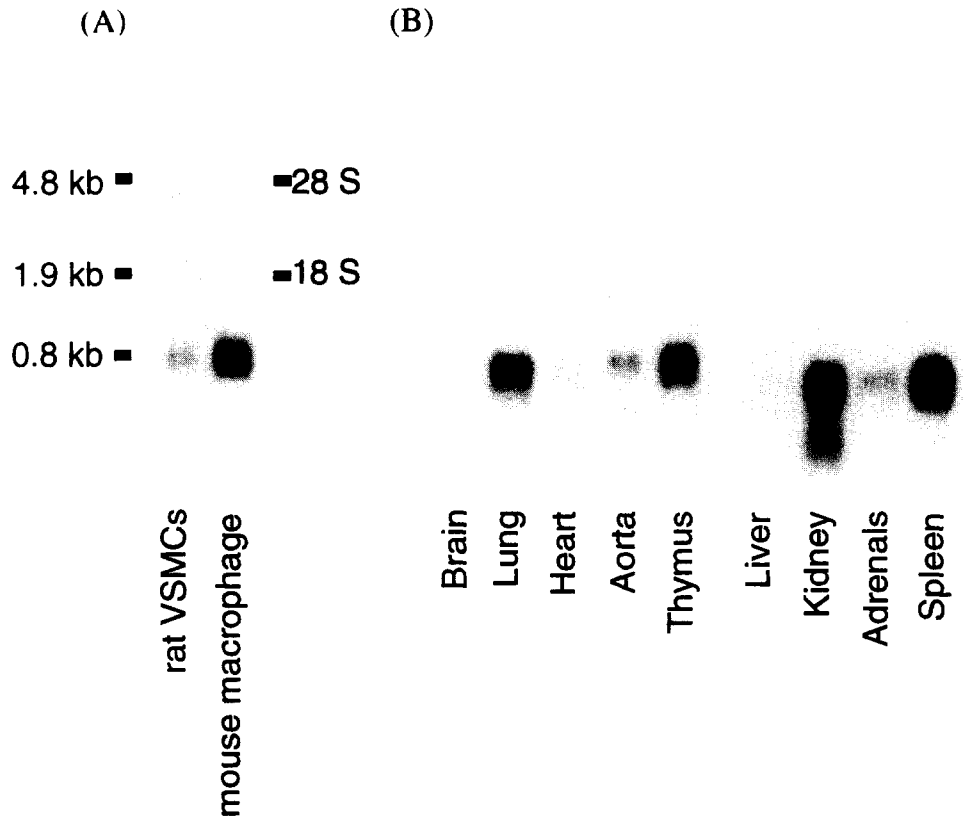

Fig. 2. Northern blotting analysis. (A) mRNA expression of the cytochrome *b*-558 α-subunit in rat VSMCs and mouse macrophage cell line, RAW 264.7. 10 μg of total RNA was loaded in each lane. (B) mRNA expression in various rat tissues. Twenty μg of total RNA was loaded in each lane. Samples were separated by electrophoresis on 1% denaturing formaldehyde agarose gels and transferred to nylon membranes. Blots were probed with the whole length rat VSMC cDNA of the cytochrome *b*-558 α-subunit.

sequence, and 96% with mouse and 89% with human in the deduced amino acid sequence. In the human neutrophil, 2 or 3 mol of heme can be associated with a heterodimer of cytochrome *b*-558 [21]. A histidine residue (His-94) has been proposed to be the heme-carrying site, since the region which contains His-94 has homology with the heme-carrying region of mitochondrial cytochrome c oxidase [18]. In the deduced amino acid sequence of rat cDNA, not only is His-94 conserved, but also the sequence containing His-94 is in good agreement with those of the possible heme-carrying regions in the human and mouse. There are two protein kinase C phosphorylation consensus sequences: KXXS (X = any amino acid residue) at position 60–63, and TXK (X = hydrophobic residue) at position 147–149 [22]. These sequences are also present in the human and mouse. The amino acid sequence of the N-terminal half was well conserved in rat, mouse and human cDNAs. Twelve amino acid residues in the C-terminal region from Gln-181 to Val-192 were also well conserved in the three species, whereas the preceding sequence from Ala-170 to Gln-181 showed the least homology among human, rat and mouse. A deletion of 3 amino acids in the rat and mouse sequences was found in the latter region. Amino acid mutations (e.g., Arg-90 → Gln, Ser-118 → Arg, Pro-156 → Gln and His-94 → Arg) that have been found in chronic granulomatous disease patients [23–25] were not found in the deduced amino acid sequence of rat cDNA.

Fig. 2A shows Northern blotting analysis of rat VSMC and mouse macrophage mRNA. A single band of 0.8 kb was observed in mRNA of rat VSMCs. This molecular size is in good agreement with those previously reported in human and mouse mRNAs. The relative level of expression of this mRNA was lower in rat VSMCs than in mouse macrophages. Fig. 2B shows the tissue distribution of cytochrome *b*-558 α-subunit mRNA expression in rat. This mRNA was expressed not only in lymphocytic tissues such as spleen and thymus, but also in non-lymphocytic tissues including aorta, lung, kidney, adrenals, and heart. The relative expression level of mRNA was considerably higher in kidney and lung than in aorta, adrenals, and heart. In contrast, mRNA expression was not detectable in liver or brain. The same 0.8 kb band was observed in all rat tissues except the kidney which also expressed a smaller detectable band.

Fig. 3 shows the results of Southern blotting analysis of rat genomic DNA. After digestion by restriction enzymes which do not cleave the cDNA sequence of rat cytochrome *b*-558 α-subunit, genomic DNA was probed under low stringency condition. Each digested sample showed a single band, except samples cut by *Hin*dIII or a combination of *Hin*dIII and *Bam*HI. The fact that *Hin*dIII-cut samples showed two bands may indicate that the gene has one restriction site in an intron. These findings suggest that only one gene encodes the cytochrome *b*-558 α-subunit in rat, which is compatible with the observation that human

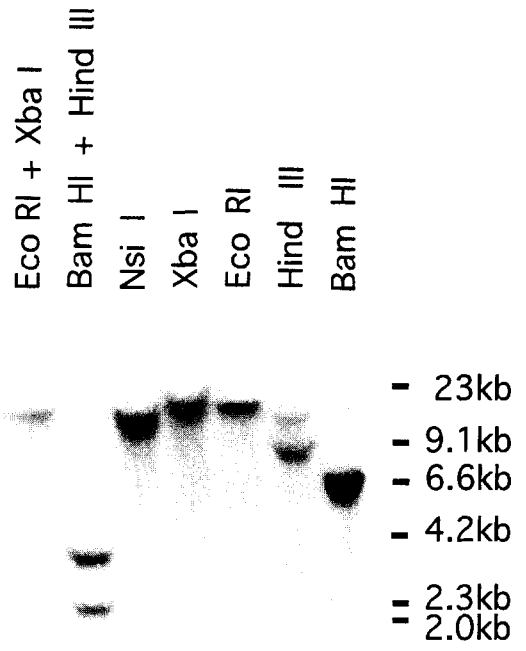

Fig. 3. Southern blotting analysis. Genomic DNA was purified from rat VSMCs with the QIAamp tissue kit (QIAGEN). 20 μg genomic DNA of rat VSMCs was digested with *Bam*HI, *Hin*dIII, *Eco*RI, *Xba*I or *Nsi*I, or a combination of *Bam*HI and *Hin*dIII or *Eco*RI and *Xba*I. The digested samples were separated on a 0.7% agarose gel and transferred to a nylon membrane. Blots were probed with the whole-length rat VSMC cDNA of the cytochrome *b*-558 α-subunit.

cytochrome *b*-558 α-subunit is derived from a single locus at 16q24 [23].

In the present study, we have shown that the rat cDNA of the cytochrome *b*-558 α-subunit conserved high homology with those of human and mouse, and that the mRNA of the cytochrome *b*-558 α-subunit was expressed in rat VSMCs as well as rat non-phagocytic tissues. Although earlier studies have suggested that the β-subunit of cytochrome *b*-558 is important to the stability of both α-and β-subunits, and to the catalytic activity of enzyme as a whole [18,26,23], the function of the cytochrome *b*-558 α-subunit is not well understood, especially in non-phagocytic cells. Future studies will be focused on the exploration of the functional consequences of mRNA expression of the cytochrome *b*-558 α-subunit and its role in superoxide anion generation in VSMCs in both normal and pathological states.

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