Identification of oxidative stress-regulated genes in rat aortic smooth muscle cells by suppression subtractive hybridization

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Abstract **A suppression subtractive hybridization technique was used to identify reactive oxygen species (ROS)-regulated genes in rat vascular smooth muscle cells. Three genes out of 89 clones, identified as fibronectin, p105 coactivator and ECA39, showed increased expression after treatment with H_2O_2. The mRNA expressions of these three genes were induced in a time- and dose-dependent manner, independent of protein kinase C activation. Immunohistochemical staining showed that the p105 coactivator expression was markedly induced in the neointima of balloon-injured rat carotid arteries. These results suggest that ROS may play an important role in the development of atherosclerosis by regulating the gene expressions we identified in this study.**

Key words: Atherosclerosis; Oxidative stress; Smooth muscle cell

1. Introduction

Recently, there has been a growing interest in the potential role of reactive oxygen species (ROS) in the pathogenesis of cardiovascular disease. Experimental animal studies have indicated that increased ROS production is associated with risk factors of atherosclerosis such as hypertension [1], hypercholesterolemia [2] and diabetes [3]. Indeed, clinical mass studies [4,5] have also provided support for the significance of ROS in the development of atherosclerosis. Induction of ROS causes an increase in peroxidized lipids such as oxidized LDL and thus enhances the foam-cell formation of macrophages [6] infiltrating into the subendothelial space and promotes migration of vascular smooth muscle cells (VSMC) [7]. In addition to such a macrophage-mediated mechanism, ROS can directly stimulate DNA synthesis and accelerate proliferation of VSMC [8], which plays a key role in the development of atherosclerosis. Although, it has been demonstrated using the candidate gene approach that ROS are potential modulators of growth factor gene expressions in VSMC: among the targets of ROS activation are heparin-binding epidermal growth factor-like growth factor [9], insulin-like growth factor I [10] and several growth-related proto-oncogenes including c-myc and c-fos [11], the overall examination of the genes implicated in ROS-mediated atherosclerosis. Thus, ROS seem to be involved in the development of atherosclerosis through a complicated pathway but the details of the mechanisms remain to be clarified.

In this study, to make an approach to the mechanism for ROS-mediated atherosclerosis, we sought to identify ROS-induced gene expressions in VSMC using the suppression subtractive hybridization (SSH) technique [12].

2. Materials and methods

2.1. Cell culture

VSMC were isolated from the thoracic aortas of 200–250 g Sprague-Dawley rats as described previously [13]. Cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37°C. Cells at passages 5–15 were used for experiments.

2.2. Isolation of poly(A)+RNA

Polyadenylated RNA was isolated using Oligotex-dT30 < Super > (Nippon Rosche, Japan Synthetic Rubber, Japan) according to the recommendations of the manufacturer.

2.3. Generation of a subtracted library by SSH

SSH was performed using the Clontech PCR-Select cDNA Subtraction kit (Clontech Laboratories, Palo Alto, CA, USA) following the manufacturer's recommendations. Starting material consisted of 2 μg H_2O_2-stimulated VSMC mRNA ('tester') and 2 μg non-stimulated VSMC mRNA ('driver'). Products from the secondary polymerase chain reactions (PCRs) were inserted into pCR 2.1 using a T/A cloning kit (Invitrogen, San Diego, CA, USA).

2.4. Northern blot analyses

Northern blot analysis followed standard procedures [14] with some modifications. Total RNA was isolated from VSMC before and after 3 h treatment with 200 μM H_2O_2. Twenty μg of the total RNA was size-fractionated by 1.2% agarose gel electrophoresis and transferred to a nylon membrane. The probes used were ^{32}P-labelled cloned cDNA inserts which were released from the pCR 2.1 vector by *Eco*RI digestion. The 950 bp *Nco*I fragment of mouse c-fos cDNA, which was kindly provided from Dr Syuichi Hirai (Yokohama City University, Yokohama, Japan) was also used as a probe. After hybridization with the labelled probes at 42°C, the membrane was washed twice with 2×SSC (SSC: 1×SSC, 15 nM sodium citrate, 150 nM NaCl, pH 7.5), which contained 0.1% sodium dodecyl sulfate at 50°C for 60 min, and was then washed with 0.2×SSC and 0.1% SDS at 50°C for 30 min. Autoradiography was performed using an intensifying screen at −80°C and the exposure time was varied so that the band intensity was kept within the linear range.

2.5. Sequencing analysis

DNA sequencing analyses were performed by the dideoxynucleotide chain termination method [15] using an Applied Biosystems Model 310 automated DNA sequencer. Gene database searches were performed through the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD, USA) using the BLAST network service.

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Abbreviations: ROS, reactive oxygen species; SSH, suppression subtractive hybridization; VSMC, vascular smooth muscle cells

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2.6. Balloon arterial injury of rat carotid artery

Male Sprague-Dawley rats (400 g, 14–16 weeks old) were anesthetized with an intraperitoneal injection of sodium pentobarbital (45 mg/kg). The bifurcation of the left common, internal and external carotid arteries was temporarily ligated. A 2F balloon catheter (Baxter Edwards Healthcare, Irvine, CA, USA) was introduced into the external carotid artery and advanced to the distal ligation of common carotid artery. The catheter was passed three times with the balloon inflated with saline to distend the common carotid artery. The catheter was then withdrawn and the proximal external carotid artery was ligated and the wound was closed. The right carotid arteries were not damaged and served as controls. The animal studies were conducted in accordance with the 'Principles of laboratory animal care' in NIH publication no. 85-23.

2.7. Immunohistochemistry

The carotid arteries were fixed with 10% phosphate-buffered neutral formalin by perfusion at 120 mm Hg pressure via the left ventricle at 14 days. After 5 min perfusion, the entire right and left carotid arteries were removed and then fixed overnight by immersion in the same fixative at 4°C. The tissues were then washed twice with phosphate-buffered saline (PBS). The middle third segment of injured or uninjured common carotid artery was excised and dehydrated in ethanol series for paraffin embedding and approximately 5 μm sections were prepared and mounted on slides. Before each incubation with antibodies described below, the mounted sections were rinsed with PBS three times. Immunohistochemical staining was performed using an avidin-biotin-peroxidase kit (Vectastastain Elite ABC kit, Vector Laboratories). The mounted sections were incubated with rabbit antiserum against human p100 coactivator (a homologue of rat p105 coactivator), which was generously provided by Dr Elliott Kieff (Harvard University, Boston, MA, USA) diluted 1:50 in PBS containing 1% BSA for 30 min. This was followed by incubation with biotinylated anti-rabbit IgG (Vector Laboratories), diluted 1:200 for 30 min, used as the secondary antibody. The sections were then incubated with ABC reagent for 30 min and positive reactions were visualized by incubation with the peroxidase substrate solution containing 3,3′-diaminobenzidine tetrahydrochloride (Zymed Laboratories, San Francisco, CA, USA).

3. Results

The SSH technique was utilized to identify ROS inducible genes in VSMC. Messenger RNAs used for the SSH were isolated from cultured VSMC before and after 3 h treatment with 200 μM H_2O_2. A series of subtractive PCR amplification and subsequent cloning into the plasmid vector (pCR 2.1) yielded 147 cDNA clones. By comparison of the length of the inserted cDNA fragments, 89 putatively independent cDNA clones were identified. To determine whether or not the expression of the cloned cDNA (gene) is increased by H_2O_2, we performed Northern blot analyses. Using each isolated cDNA clone as a probe, eight cDNAs (genes) out of 89 were found to be activated in response to H_2O_2 (Fig. 1). DNA sequencing analyses for those eight cDNA clones identified two as fibronectin and p105 coactivator (accession number U83883). Another clone showed a high similarity with nucleotide sequences to a mouse cDNA, ECA39 (79/84, 94%), and was therefore judged as encoding the rat counterpart for ECA39.

For the other five clones activated with H_2O_2 (arbitrarily termed as clones A–E), sequence similarities were only found in the so-called expression sequence tags (ESTs). Clones A and B matched previously reported ESTs in rat (A, accession number C07195; B, C06962). Clones C and D were similar to mouse ESTs (C, 96% (178 out of 186 nucleotides) homologous to AA032677; D, 98% (194 out of 198) to AA119849). Clone E was similar to a human EST (82% homologous to AA213802). Thus, clones C, D and E were considered to be the rat counterparts of those cDNAs.

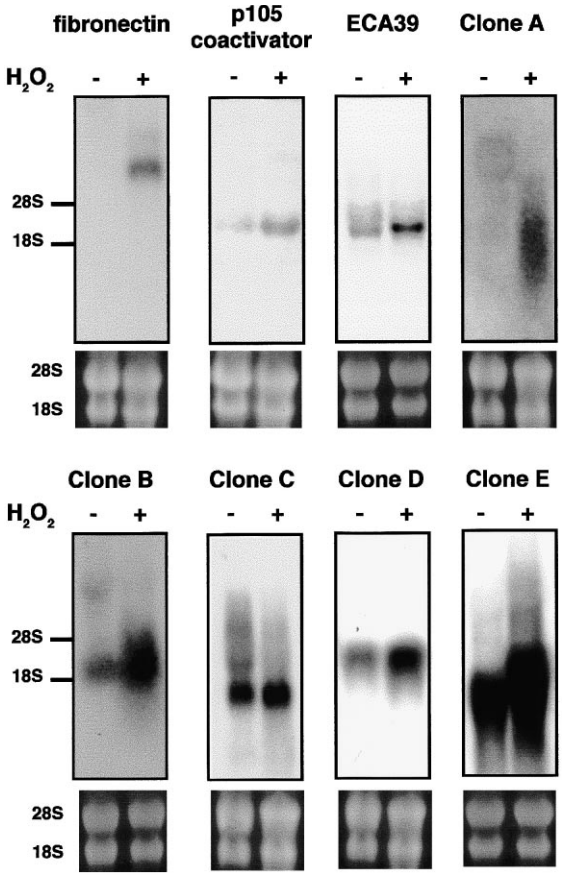

Fig. 1. Effect of H_2O_2 on fibronectin, p105 coactivator and ECA39 mRNA expressions in VSMC. Northern blot analyses were performed using 20 μg total RNA isolated from VSMC before and after 3 h treatment with 200 μM H_2O_2. Similar results were obtained with three independent experiments.

Induction of fibronectin, p105 coactivator and ECA39 mRNA expressions was observed in a time-dependent manner. After addition of H_2O_2, the level of fibronectin mRNA increased at 0.5 h and returned to the near base line after 6 h. The levels of p105 coactivator and ECA39 mRNA increased at 3 h and there was a sustained increase of the levels of p105 coactivator and ECA39 mRNA until after 24 h (Fig. 2A). Also, H_2O_2-induced fibronectin, p105 coactivator and ECA39 mRNA expressions were observed in a dose-dependent manner (Fig. 2B).

Because in several cell types including VSMC, oxidant-mediated activation of protein kinase C (PKC) has been reported [16–18], we examined if PKC is implicated in the ROS-regulated expression of those three genes. VSMC were preincubated with 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 24 h, during which PKC is down-regulated by persistent treatment with TPA [19]. After this pretreatment, the cells were treated with 200 μM H_2O_2 for the indicated time. The results were shown in Fig. 2C. To confirm complete suppression of PKC activity by TPA pretreatment, we demonstrated that TPA pretreatment completely blocked induction of c-fos mRNA by phorbol 12,13-dibutyrate (PDBU), which was reported to be induced by PKC-dependent mechanisms [11,20]. TPA pretreatment had no effect on the accu-

mulation of fibronectin, p105 coactivator and ECA39 mRNA by H_2O_2. Thus, it seems that ROS-induced activation of these three genes is not mediated by PKC activation.

To examine whether the ROS-regulated genes we identified are implicated in the development of atherosclerosis, immunohistostaining for p105 coactivator was performed in balloon-injured rat carotid arteries. At 14 days after injury, the intima was markedly thickened and p105 coactivator expression was markedly induced in the neointima of injured arteries as compared with uninjured control (Fig. 3). No immunoreactivity can be seen with normal rabbit serum in the left carotid artery 14 days after injury (data not shown).

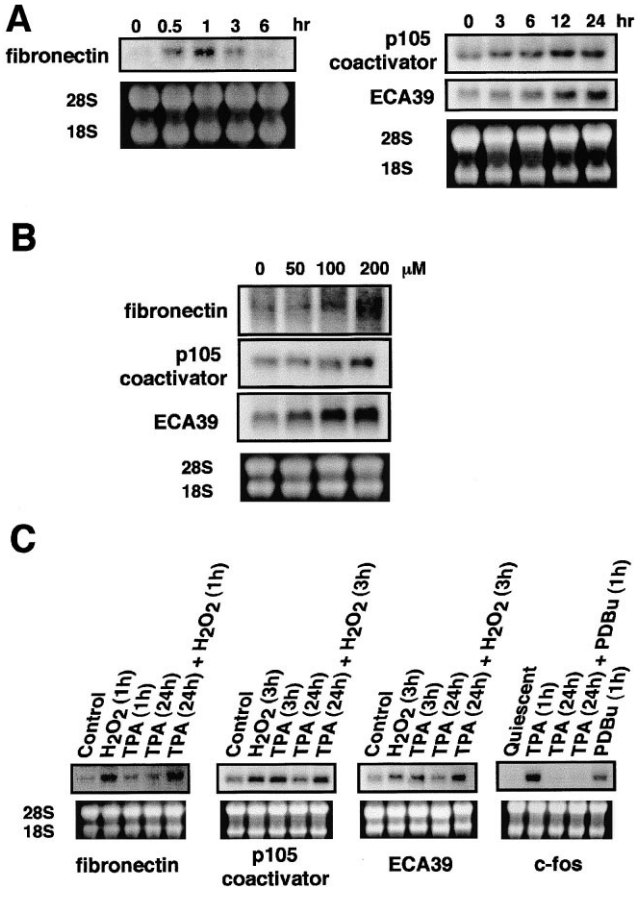

Fig. 2. (A) Time course of H_2O_2-induced fibronectin, p105 coactivator and ECA39 mRNA expressions in VSMC. Total RNA was isolated from VSMC treated for the indicated times with 200 μM H_2O_2 and Northern blot analyses were performed. (B) Dose-dependent induction of fibronectin, p105 coactivator and ECA39 mRNA expressions in VSMC. Total RNA was isolated from VSMC treated with 0, 50, 100 or 200 μM H_2O_2 for 3 h and Northern blot analyses were performed. (C) Effect of TPA pretreatment on H_2O_2-induced fibronectin, p105 coactivator and ECA39 mRNA expressions. For the evaluation of fibronectin, p105 coactivator and ECA39 mRNA expressions, VSMC were treated as follows (lane 1–5, from left to right): lane 1, no additions; lane 2, H_2O_2 (200 μM) alone for 1 h (fibronectin) or 3 h (ECA39 and p105 coactivator); lane 3, TPA (100 nM) alone for 1 h (fibronectin) or 3 h (ECA39 and p105 coactivator); lane 4, TPA (100 nM) alone for 24 h; lane 5, TPA (100 nM) alone for 24 h, followed by H_2O_2 (200 μM) for 1 h (fibronectin) or 3 h (ECA39 and p105 coactivator). For the evaluation of c-fos mRNA expression as a control, VSMC were made quiescent by incubation in fresh DMEM containing 0.4% FCS for 48 h before TPA pretreatment. VSMC were treated as follows (lane 1–5, from left to right): lane 1, quiescent, no additions; lane 2, TPA (100 nM) alone for 1 h; lane 3, TPA (100 nM) alone for 24 h; lane 4, TPA (100 nM) alone for 24 h, followed by PDBU (200 nM) 1 h; lane 5, PDBU (200 nM) alone for 1 h. Total RNA was isolated from VSMC and Northern blot analyses were performed. Similar results were obtained with three independent experiments.

4. Discussion

Proliferation of VSMC constitutes a key step in the development of atherosclerosis. Although various growth factors and oncogenes have been suggested to be involved in the regulation of VSMC proliferation, the overall mechanism for the development of atherosclerosis remains poorly elucidated. Recently, ROS have been found to be related to VSMC proliferation. The induction of ROS has often been observed in vivo under certain conditions such as hypertension [1], hypercholesterolemia [2] and diabetes [3] and recent data suggest that ROS may play an important role in the development of atherosclerosis. In this study, we focused on oxidative stress as a cause of atherosclerosis and sought to identify factors which may mediate ROS-induced VSMC proliferation. Identification of the factors involved in the oxidative stress-mediated atherosclerosis may lead to understanding of the mechanism for the development of atherosclerosis and enable efficient prevention.

The pathophysiological significance of the three ROS-induced genes newly identified in this study remains to be clarified. Previous studies revealed that fibronectin promotes the migration of VSMC and its mRNA level is increased in atherosclerotic lesion [21]. It was also reported that the fibronectin gene expression is induced in the smooth muscle cells in the balloon injury model in which large amount of ROS are produced [22]. Therefore, as the fibronectin gene is activated in response to ROS in VSMC, ROS-mediated fibronectin expression is likely to explain in part the cause of atherosclerosis. Here, we would like to note that the ROS-responsive fibronectin gene expression was recently found in the mesangial cells of the kidney [23]. Considering the developmental, biological and functional similarities between the mesangial cells in kidney and VSMC, a similar mechanism is likely to operate in the ROS activation of the fibronectin gene in those two cells.

Rat p105 coactivator is a homologue of human p100 coactivator, which binds c-Myb and regulates its activity [24,25]. Suppression of its expression with antisense oligonucleotide has been shown to cause reduction in cell growth [26]. In the present study, the expression of p105 coactivator protein was shown in the neointima of balloon-injured rat carotid arteries. This suggests that p105 coactivator is involved in cell growth and supports the idea that ROS-activated expression of p105 coactivator may contribute to the VSMC growth and proliferation.

The ECA39 gene is known to bear a functional c-Myc-binding sequence located 3′ to its transcription start site and regulated by c-Myc [27]. Because the c-myc gene is also ROS-responsive, the ROS-induced c-myc gene activation may mediate ROS-responsive ECA39 expression. Recently, the product of ECA39 has been reported to be involved in control of the cell cycle, suppressing the G1 to S transition in the cell cycle and eventually leading to apoptosis [28]. Thus, although in this study, the expression of ECA39 protein in atherosclerotic lesion was not shown due to unavailability of ECA39 antibody, ROS-mediated ECA39 expression may explain in

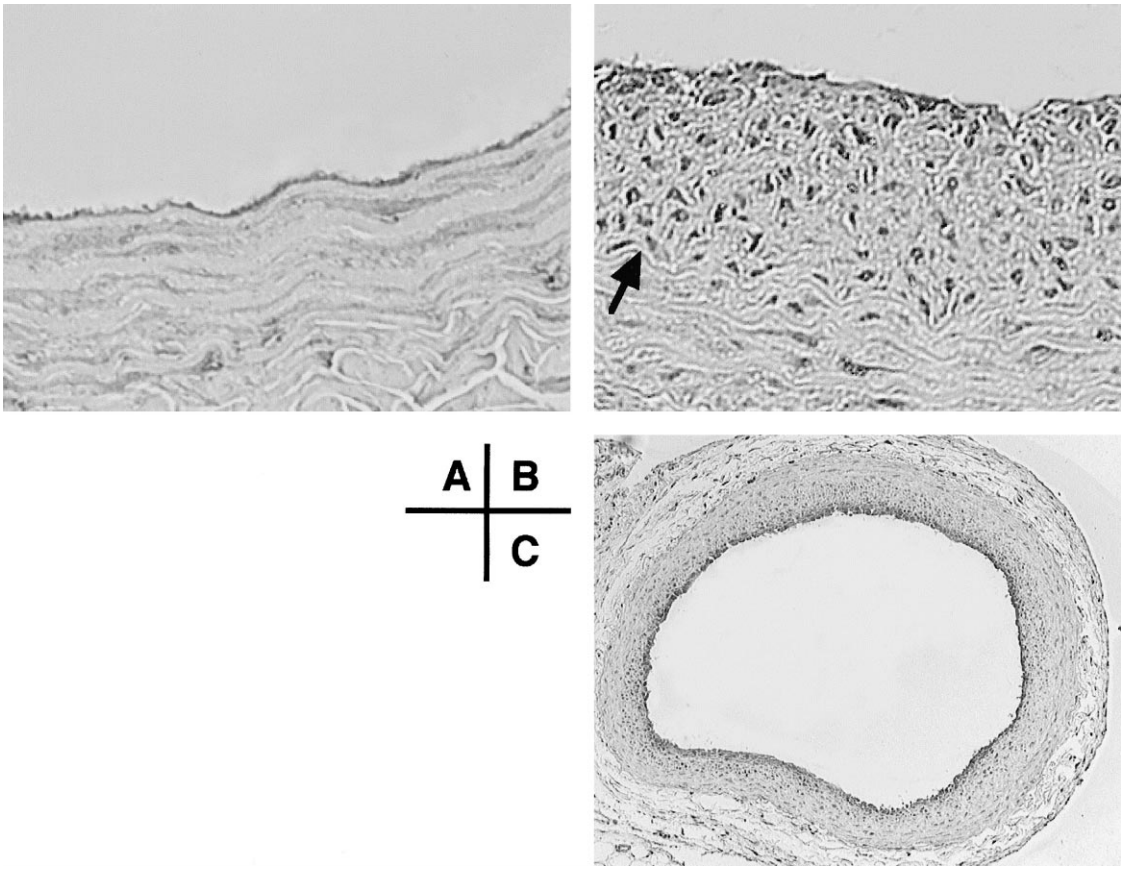

Fig. 3. Immunohistochemical localization of p105 coactivator in the neointima of balloon-injured rat carotid arteries. (A) Right uninjured carotid artery. (B) Left carotid artery 14 days after injury. The arrow marks the internal elastic lamina. p105 coactivator expression is observed in the nuclei in the neointimal cells. (C) A lower power view of the cross section of the rat carotid artery 14 days after injury shows a remarkably thickened intima and neointimal cells markedly positive for p105 coactivator immunostaining. Original magnification ×400 (A and B) and ×100 (C).

part the induction of apoptosis of VSMC, which can be observed in atherosclerotic lesions.

Because oxidant-mediated activation of PKC in VSMC has been reported and PKC has been known to play an important role in the expressions of various genes [11,20,29], we investigated whether PKC is implicated in the ROS-regulated expression of those three genes. Although the results suggested that ROS-induced activation of these three genes is not mediated by PKC (Fig. 2C), it remains to be elucidated how ROS can be the activator of the three genes identified in this study. Included among the transcription factors known to mediate ROS-responsive gene activation are AP-1 [30], NF-kB [31] and CArG box-binding factor [32]. Among these, only the AP-1-binding site sequence can be seen in the promoter region of the fibronectin gene [33]. In terms of the rat p105 coactivator and ECA39 genes, no information is available to date with respect to their promoter structures. Further efforts are needed to clarify the mechanisms underlying the ROS regulation of those genes.

In summary, we identified ROS-mediated potentially important factors for the development of atherosclerosis in VSMC by using the SSH technique. These results suggest that ROS may play an important role in the development of atherosclerosis by regulating these gene expressions.

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