Published in final edited form as:

Free Radic Biol Med. 2007 September 15; 43(6): 976–986. doi:10.1016/j.freeradbiomed.2007.06.001.

Nox2 regulates endothelial cell cycle arrest and apoptosis via p21^{cip1} and p53

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Abstract

Endothelial cells (EC) express constitutively two major isoforms (Nox2 and Nox4) of the catalytic subunit of NADPH oxidase, which is a major source of endothelial reactive oxygen species. However, the individual roles of these Noxes in endothelial function remain unclear. We have investigated the role of Nox2 in nutrient deprivation-induced cell cycle arrest and apoptosis. In proliferating human dermal microvascular EC, Nox2 mRNA expression was low relative to Nox4 (Nox2:Nox4 ~1:13), but was upregulated 24 h after starvation and increased to 8 ± 3.5 -fold at 36 h of starvation. Accompanying the upregulation of Nox2, there was a 2.28 ± 0.18 -fold increase in O₂^{•-} production, a dramatic induction of p21^{cip1} and p53, cell cycle arrest, and the onset of apoptosis (all $p < 0.05$). All these changes were inhibited significantly by in vitro deletion of Nox2 expression and in coronary microvascular EC isolated from Nox2 knockout mice. In Nox2 knockout cells, although there was a 3.8 ± 0.5 -fold increase in Nox4 mRNA expression after 36 h of starvation ($p < 0.01$), neither O₂^{•-} production nor the p21^{cip1} or p53 expression was increased significantly and only 0.46% of cells were apoptotic. In conclusion, Nox2-derived O₂^{•-}, through the modulation of p21^{cip1} and p53 expression, participates in endothelial cell cycle regulation and apoptosis.

Keywords

NADPH oxidase; Endothelial cells; Cell cycle; p21^{cip1}; p53; Apoptosis; ROS

Endothelial cells (EC) respond to changes in hemodynamic forces, ambient pO_2 , and local signals with appropriate functional changes to maintain homeostasis and neovascularization [14,27,29]. To achieve this, the endothelium has to be stable (highly quiescent) and also be able to undergo angiogenesis (proliferation) or remodeling (apoptosis) when required. Normal EC function and survival require a consistent supply of nutrients and growth factors. During ischemia, angioplasty, or organ transplantation, endothelial cells experience sustained nutrient deprivation (starvation), which causes EC injury or apoptosis. Although increased reactive oxygen species (ROS) production and oxidant stress have been found to be involved in nutrient deprivation-induced EC injury, the enzymatic sources of ROS production and redox-signaling pathways are far from clear. Understanding the mechanisms and the signaling pathways underlying nutrient deprivation-induced EC oxidative stress might help us to prevent EC death and to regenerate damaged endothelium. Recently, it was

reported that ROS generated from an NADPH oxidase may serve as signaling molecules involved in the regulation of EC growth, death, and function [1,10,28].

The NADPH oxidase is expressed constitutively in EC and represents a major source of $O_2^{\bullet-}$ generation [12,20]. It comprises one catalytic subunit of the Nox family, one $p22^{phox}$ subunit, and at least four regulatory subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and $rac1$) [26]. Under physiological conditions, EC NADPH oxidase produces low levels of ROS, which may serve as second messengers involved in cellular signaling. However, the enzyme activity can be upregulated in response to stimuli such as shear stress [4], growth factors [1], and nutrient deprivation [3]. High levels of ROS cause EC injury or apoptosis. The catalytic subunit of NADPH oxidase (Nox) has several isoforms and at least three of them (Nox1, Nox2, and Nox4) are expressed in EC. However, the precise roles of each Nox in EC growth or growth arrest, e.g., cell cycle regulation, are far from clear.

The mammalian cell cycle is tightly controlled by a series of regulatory molecules known as cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CDKIs) [16]. Among these molecules, $p21^{cip1}$ is a G_1 checkpoint inhibitor leading to cell cycle arrest and the inhibition of DNA replication [9,16]. Interestingly, $p21^{cip1}$ has been found to be redox-sensitive. For example, shear stress, a strong activator of NADPH oxidase, increased $p21^{cip1}$ expression and thereafter suppressed the EC G_1 - to S-phase transition [4]. Low-density lipoproteins derived from diabetic patients were found not only to cause EC oxidant stress but also to induce $p21^{cip1}$ expression and abnormal G_1 -phase accumulation [5]. It is therefore possible that $p21^{cip1}$ might sense the redox states in EC and control cell cycle progression. The tumor suppressor gene p53 also is inducible by oxidative stress and plays an important role in cell cycle regulation. Once it is induced, p53 can also act as a transcriptional activator for $p21^{cip1}$ [25].

In this study, we have investigated the roles of Nox2 and Nox4 (the two major Nox isoforms expressed in EC) in nutrient deprivation-induced oxidative stress and the potential downstream molecules in cell cycle regulation. We found that ROS derived from Nox2 (but not Nox4) are functionally involved in the regulation of the cell cycle inhibitors $p21^{cip1}$ and p53 and participate in EC cell cycle regulation and apoptosis.

Materials and methods

Reagents

Culture medium, fetal calf serum (FCS), glutamine, and antibiotics were purchased from Gibco BRL (UK). Collagenase type II, EC growth supplement, gelatin, trypsin, DNase, RNase, lucigenin, NADPH, diphenyleneiodonium (DPI), tiron, apocynin, oxypurinol, rotenone, *N*- ω -nitro-L-arginine methyl ester (L-NAME), and propidium iodide were from Sigma (UK). Dihydroethidine and 5-(and 6)-chloromethyl-2',7'-dichlorofluorescein diacetate (DCF) were from Molecular Probes (UK). Goat polyclonal antibodies against gp91^{phox}, rabbit polyclonal antibodies to Nox4, p47^{phox}, p21^{cip1}, and p53 were purchased from Santa Cruz Biotechnology (UK). All other reagents were from Sigma (UK).

Coronary microvascular endothelial cell (CMEC) isolation and cell culture

The colonies of Nox2 (gp91^{phox}) knockout mice on a C57BL6 background were purchased from The Jackson Laboratory (USA) and bred in our school. All studies conformed with the *Guidance on the Operation of Animals (Scientific Procedures) Act*, 1986 (UK). CMEC were isolated from the hearts of 10- to 12-week-old male Nox2 KO and age-matched wild-type mice according to the method previously reported [17]. Six animals from each group were used for each isolation. Human dermal microvascular EC (HMEC1) were obtained from the Centers for Disease Control and Prevention (Atlanta, GA, USA) [2]. HMEC1 were cultured

in M199 and CMEC were cultured in DMEM, both growth media containing 10% FCS, EC growth supplement (30 $\mu\text{g/ml}$), epidermal growth factor (10 ng/ml), vascular endothelial growth factor (0.5 ng/ml), ascorbic acid (1 $\mu\text{g/ml}$), hydrocortisone (1 $\mu\text{g/ml}$), L-glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 $\mu\text{g/ml}$). CMEC were used at passage 2.

Cell proliferation, growth arrest, and cell cycle analysis

For cell cycle analysis, EC (fully confluent and quiescent) were counted and seeded at $2 \times 10^5/\text{ml}$ in 100- mm^2 cell culture dishes (3 ml) for 4–6 h to settle down and to achieve ~70% of cell confluence. The cells were then used immediately (0-h sample) or cultured in complete growth medium (proliferating) or in medium supplemented with 0.2% serum (starvation) for 12, 24, and 36 h. Serum was used at 0.2% to maintain cell viability and good morphology. Cells were then detached with trypsin/EDTA, counted, and used immediately for cell cycle analysis as described previously [4,11]. Briefly, EC were fixed with 70% ethanol, treated with RNase A (0.2 mg/ml), and then stained with propidium iodide (50 $\mu\text{g/ml}$) for DNA content. The number of cells in G_0/G_1 , S, and G_2/M phases and apoptotic cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

Quantitative real-time PCR

Total RNA was prepared with a Qiagen kit. RNA (1 μg) was reverse-transcribed by AMV transcriptase (Promega) in a total volume of 20 μl . The cDNA (0.5 μl) was amplified with a sequence detection system (ABI Prism 7000) in a total volume of 25 μl containing 12.5 μl of SYBR green PCR master mix (ABI) and each primer at 0.1 μM . Human primers used were Nox2, (F) CAAGATGCGTGGAACCTACCTAAGAT and (R) TCCCTGCTCCCACTAACATCA; Nox4, (F) CTGCTGACGTTGCATGTTTC and (R) TTCTGAGAGCTGGTTCGGTT; p47^{phox}, (F) CCACCTCCTCGACTTCTTCA and (R) TGTCTGTCGCGGTACTCTTG; p21^{cip1}, (F) GGACAGCAGAGGAAGACCATGT and (R) TGGAGTGGTAGAAATCTGTCATGC; and β -actin, (F) CTGGCACCCAGCACAATG and (R) GCCGATCCACACGGAGTACT. Mouse primers used were Nox2, (F) ACTCCTTGGAGCACTGG and (R) GTTCCTGTCCAGTTGTCTTCG; Nox4, (F) TGAACCTACAGTGAAGATTTTCCTTGAAC and (R) GACACCCGTCAGACCAGGAA; p21^{cip1}, (F) TTGTCGCTGTCTTGCACTCT and (R) AATCTGTCAGGCTGGTCTGC; β -actin, (F) CGTGAAAAGATGACCCAGATCA and (R) TGGTACGACCAGAGGCATACAG; and GAPDH (F) CGTGCCGCTGGAGAA and (R) CCCTCAGATGCCTGCTTCAC. Each PCR was performed in duplicate and repeated with at least three independent cell cultures or CMEC isolations. The PCR products of each gene had been sequenced. The expression levels were standardized using two housekeeping genes, GAPDH and β -actin, and the final results were expressed as a molar/molar ratio relative to β -actin.

ROS production

NADPH (100 μM)-dependent $\text{O}_2^{\bullet-}$ production by EC homogenates was assessed by lucigenin (5 μM)-enhanced chemiluminescence (BMG Lumistar, Germany). The specificity of the assay for $\text{O}_2^{\bullet-}$ was confirmed by adding tiron (5 mM), a nonenzymatic scavenger of $\text{O}_2^{\bullet-}$, to inhibit the $\text{O}_2^{\bullet-}$ -dependent chemiluminescence. Potential sources of $\text{O}_2^{\bullet-}$ production were assessed using: (a) apocynin (10 μM) to inhibit NADPH oxidase; (b) L-NAME (0.1 mM) to inhibit $\text{O}_2^{\bullet-}$ production by dysfunctional NO synthase; (c) oxypurinol (100 μM) to inhibit xanthine oxidase; (d) DPI (20 μM) to inhibit flavoprotein enzymes; and (e) rotenone (50 μM) to inhibit mitochondrial ROS production. Each sample was tested in triplicate to take the mean. Data were expressed as mean arbitrary light units per milligram of protein per minute over a 20-min period, and the final results were shown as the mean $\pm$ SD from at least three separate cell cultures. In some experiments, ROS generation by intact CMEC was measured by DCF fluorescence [15]. Briefly, adherent cells cultured on

chamber slides were washed in Hanks' buffer and then incubated with 10 μ M DCF in Hanks' buffer for 30 min at room temperature. DCF fluorescence at an excitatory wavelength of 495 nm was acquired on a Zeiss LS510 confocal microscopy system. Fluorescence intensity was quantified from at least three random fields (1024×1022 pixels; $269.7 \times 269.2 \mu\text{m}$) per slide (100 cells assessed per slide) and three slides per experimental condition.

Generation of full-length human Nox2 antisense cDNAs and gene transfection

The full-length human Nox2 cDNA was inserted in the antisense orientation into the expression vector pcDNA3.1 (Invitrogen) and confirmed by sequencing. Transfection was undertaken with Lipofectamine 2000 and Plus reagent (Gibco) in serum-free medium as described previously [18]. The transfection medium was prepared by mixing 3 $\mu\text{g}/\text{ml}$ cDNA construct with 5 $\mu\text{l}/\text{ml}$ Plus reagent and incubating at room temperature for 15 min. Lipofectamine 2000 (final concentration 5 $\mu\text{g}/\text{ml}$) was added to the mixture and incubated for a further 15 min at room temperature. Cells were incubated in transfection medium for 4 h at 37°C in 95% air/5% CO_2 , after which 10% FCS was added to the medium and incubated with cells overnight. The next day, the medium was replaced with complete growth medium and cells were used for experiments 48 h after transfection.

Immunoblotting

Cells were lysed directly in SDS sample buffer containing Tris-HCl (50 mM) and SDS 1% (pH 7). Cell lysates were extracted on ice for 15 min and centrifuged at $200g$ for 5 min at 4°C to remove the nuclei. Soluble protein concentration was determined using a Bio-Rad kit (Bio-Rad Laboratories, UK). Immunoblotting (25 μg protein per sample) was performed as described previously using antibodies (1:1000 dilution) against Nox2, p47^{phox}, p21^{cip1}, p53, and α -tubulin [19]. The expression of α -tubulin was used as a loading control.

Immunofluorescence confocal microscopy

Confocal microscopy was performed as described previously [19]. Briefly, endothelial cells were cultured onto four-chamber slides precoated with 1% gelatin. Cells were permeabilized and fixed with methanol/acetone (50% each, v/v). Slides were blocked with 20% FCS in PBS for 30 min at room temperature. Cells were washed with 0.1% BSA/PBS three times with gentle shaking and then incubated with the primary antibodies diluted (1:250 to 1:1000) in 0.1% BSA/PBS for 30 min at room temperature. Biotin-conjugated anti-rabbit or anti-goat IgG (1:1000 dilution) was used as the secondary antibody and incubated for 30 min. Specific antibody binding was detected by FITC (green fluorescence)-labeled extravidin. Normal rabbit or goat IgG (5 $\mu\text{g}/\text{ml}$) was used instead of primary antibody as negative control in each case. Confocal microscopy was performed using a Zeiss LS510 confocal microscopy system. Optical sections were taken at 0.5 μm intervals, and images were captured and stored digitally for analysis. Fluorescence intensity was quantified from at least three random fields (1024×1022 pixels; $269.7 \times 269.2 \mu\text{m}$) per slide, three slides per experimental condition, and repeated three times using separate cell cultures.

Statistics

Data are presented as means $\pm$ SD from at least three experimental results taken from three independent cultures for each condition. Comparisons were made by unpaired *t* test, with Bonferroni correction for multiple testing. A $p < 0.05$ was considered statistically significant.

Results

Time course of nutrient deprivation-induced cell growth arrest, apoptosis, and ROS generation

A time course of nutrient deprivation was performed using a human dermal microvascular EC line. Cells were starved in 0.2% FCS medium that maintains cell viability and good morphology in the absence of cell proliferation. Cells were assessed at 0, 12, 24, and 36 h for changes in cell number (Fig. 1A), apoptosis (Fig. 1B), and NADPH-dependent $O_2^{\bullet-}$ production (Fig. 1C).

Compared to the cell number at 0 h, cells proliferated initially within 12 h of starvation ($39 \pm 0.2\%$ increase in cell number), then proliferation slowed down with no further increase in cell number after 24 h of starvation (Fig. 1A). At 36 h of starvation there was significant cell apoptosis ($7.57 \pm 1.13\%$) (Fig. 1B, $p < 0.01$). We then examined ROS production in these cells. We found that within 12 h of starvation, ROS generation was actually reduced (Fig. 1C), which might reflect a reduced metabolic rate in cells under starvation. The levels of ROS were rapidly increased after 24 h of starvation and at 36 h of starvation there was 2.28 ± 0.18 -fold the levels at 0 h ($p < 0.05$), concomitant with the remarkable increase in cell apoptosis. The large amount of ROS production at 36 h could be completely inhibited by adding DPI (a flavoprotein inhibitor), apocynin (NADPH oxidase inhibitor), or tiron (an $O_2^{\bullet-}$ scavenger), but not by inhibitors of xanthine oxidase (oxypurinol), mitochondrial oxidases (rotenone), nor nitric oxide synthase (L-NAME) (Fig. 1D). These data strongly suggest that NADPH oxidase might be responsible for the high level of ROS production observed at 36 h of starvation.

Expression of mRNAs for Nox1, Nox2, Nox4, p47^{phox}, and p21^{cip} during starvation

In order to find out which Nox isoform was involved in starvation-induced oxidative stress, we examined the levels of mRNA expression of Nox1, Nox2, and Nox4 in HMEC1 by quantitative real-time PCR (Fig. 2).

Nox1 mRNA was hardly detectable in EC at 0 h and was below the level of detection after starvation. Nox4 was the predominant Nox isoform in HMEC1 at 0 h (mRNA Nox1: Nox2:Nox4 ~ 1:14:184). Nox4 mRNA was moderately increased during starvation, and at 36 h of starvation, it was 1.89 ± 0.2 times the level at 0 h ($p < 0.05$). Compared to Nox4, Nox2 mRNA expression was low at 0 h (~1/13 of Nox4) and stayed low until 24 h of starvation. Nox2 mRNA increased rapidly after that and at 36 h it was 8 ± 3.5 times the level at 0 h (Fig. 2). Although Nox4 expression remained high in cells under starvation, Nox2 was the Nox isoform largely upregulated by nutrient deprivation. Accompanying the robust elevation of Nox2, the regulatory subunit p47^{phox} was also upregulated. At 36 h, p47^{phox} mRNA was 7.2 ± 2.8 times the level at 0 h (Fig. 2). Interestingly, mRNA expression of the cell cycle inhibitory gene p21^{cip1} was increased dramatically after 24 h of starvation, and at 36 h it was 41.1 ± 5 times the level at 0 h (Fig. 2).

Relationships between the expression of Nox2, Nox4, and p21^{cip1}; ROS production; and cell cycle progression

The data obtained thus far revealed distinct changes in: (i) EC oxidative stress, (ii) Nox2 and p21^{cip1} mRNA accumulation, and (iii) EC apoptosis at 36 h of nutrient deprivation. Therefore, we focused our subsequent experiments on 36 h of nutrient deprivation and compared the results to cells maintained in growth medium.

We next examined whether the changes in mRNA expression observed for the Nox2, Nox4, and p21^{cip1} genes were mirrored at the protein level. Nox2 protein expression was very low

in HMEC1 maintained in growth medium and was obviously upregulated after 36 h starvation (Fig. 3A). The increase in Nox2 protein expression was accompanied by a clear increase in p47^{phox} expression and dramatic inductions in p21^{cip1} and p53 protein expression (Fig. 3A). However, there was no significant difference for Nox4 protein expression under these conditions. We then determined the *in situ* ROS production in EC by DCF fluorescence (Fig. 3B). We found that DCF fluorescence was mainly around the perinuclear region and could be completely inhibited by adding tiron (an O₂^{•-} scavenger), indicating that the ROS detected originated from O₂^{•-} (Fig. 3B). Quantitative analysis revealed that the level of DCF fluorescence was significantly higher in cells after starvation (1.5±0.5-fold, *p*<0.05) compared to cells maintained in complete growth medium. Cell cycle analysis revealed that, apart from starvation-induced cell cycle withdrawal (G₀/G₁ phase 70.1%), 8.04% of cells were apoptotic concurrent with the high levels of ROS production at 36 h of starvation (Fig. 3C). Starvation-induced cell apoptosis was inhibited in the presence of apocynin (1 mM), an inhibitor of NADPH oxidase (Fig. 3C). All these data together indicate that ROS-derived from Nox2 are involved in nutrient deprivation-induced oxidative stress and cell apoptosis.

In vitro transient depletion of Nox2 protein expression by Nox2 antisense cDNA

In order to further investigate the role of Nox2 in starvation-induced oxidative stress and apoptosis, we depleted transiently the Nox2 expression in HMEC1 with a full-length Nox2 antisense cDNA. The transfection efficiency was confirmed by immunoblotting (Fig. 4).

In control cells transfected with an empty vector and maintained for 36 h in growth medium, the Nox2 protein expression was low. Starvation increased notably the level of Nox2 protein in these cells, which was accompanied by (i) increases in p21^{cip1} and p53 expression (Fig. 4A), (ii) an increase in NADPH-dependent ROS production (Fig. 4B), and (iii) an increase in cell death as determined by trypan blue exclusion (Fig. 4C). In contrast, in cells transfected with full-length Nox2 antisense cDNA, the Nox2 protein expression was hardly detectable when the cells were kept in growth medium and was only very weakly detectable after 36 h of starvation (Fig. 4A). After the transient depletion of Nox2 protein, there were reductions in starvation-induced p21^{cip1} and p53 expression, and there was no significant increase in ROS production (Fig. 4B) or cell apoptosis (Fig. 4C) compared to control cells transfected with an empty vector. From these results, it is clear that Nox2-derived ROS have a specific role in mediating nutrient deprivation-induced p21^{cip1} and p53 expression and EC apoptosis.

Studies of CMEC isolated from Nox2 knockout mice

Owing to the nature of HMEC1, which harbor the SV-40 large T antigen [2,25], the results from HMEC1 may not represent the actual role of Nox2 in primary EC isolated from vascular tissues. In order to further investigate the role of Nox2 in normal EC cell cycle regulation, we isolated CMEC from wild-type and Nox2 knockout mice and examined the role of Nox2 and Nox4 in cell cycle regulation in these cells.

In wild-type CMEC maintained in growth medium for 36 h, Nox2 and Nox4 were expressed at similar levels (Fig. 5A). This is in contrast to what had been observed in transformed HMEC1 (Fig. 2). Thus, immortalized HMEC1 have a higher metabolic rate and proliferation index and Nox2 expression was very low in these cells; whereas, in primary cultures of CMEC that have a low proliferation index and die after a few passages, Nox2 expression was high. Starvation increased (4.9±1.7-fold) Nox2 (but not Nox4) expression in wild-type CMEC significantly, which was accompanied by a 40% increase in NADPH-dependent O₂^{•-} production (Fig. 5B) and a 5.8±1.2-fold increase in p21^{cip1} mRNA expression (Fig. 5A). Although there was a 3.8±0.5-fold increase in Nox4 expression after 36 h of starvation in

Nox2 knockout cells, there were no significant changes in levels of $O_2^{\bullet-}$ production nor p21^{cip1} expression (Figs. 5A and 5B).

In growth medium, wild-type CMEC were actively engaged in cell cycle progression (G_0/G_1 43.4%, S 23.3%, G_2/M 27.1%, apoptotic 0.28%) (Fig. 5C), whereas Nox2 knockout CMEC showed a delay in G_1 - to S-phase entry (G_0/G_1 49.8%, S 16.3%, G_2/M 25.9%, apoptotic 0.27%). After 36 h of serum starvation, wild-type cells withdrew from the cell cycle (G_0/G_1 72.4%, S 2.4%, G_2/M 15.1%) with ~6.69% cells being apoptotic. In contrast, Nox2 knockout CMEC seemed to be resistant to starvation-induced cell cycle withdrawal, with a considerable number of Nox2 knockout cells accumulating in the G_2/M phase of the cell cycle (G_0/G_1 64.9%, S 9.1%, G_2/M 20.4%) and only 0.46% being apoptotic.

The subcellular localization and the expression of Nox2 and p21^{cip1} were examined by confocal microscopy in wild-type CMEC (Fig. 6A). Under both growth conditions (growth medium versus 0.2% FCS for 36 h), Nox2 was detected primarily in the perinuclear region. Compared to cells in growth medium, starvation increased the level of Nox2 expression, with considerable fluorescence extending out toward the periphery (Fig. 6A). p21^{cip1} expression was mainly detected near, or within, the nucleus and was increased after starvation. Under starvation, p53 (FITC, green fluorescence) was readily detectable in both the cytoplasm and the nucleus (labeled with propidium iodide, red fluorescence) in wild-type CMEC; there was evidence of nuclear fragmentation and apoptotic bodies indicative of cell apoptosis (Fig. 6B). Compared to wild-type CMEC, p53 expression was much less in Nox2 knockout CMEC and was mainly detected in the perinuclear region. Moreover, there were significantly fewer apoptotic cells in Nox2 knockout CMEC compared to dead or dying cells in the wild-type cell culture (Fig. 6B). A reduced level of expression of p53 in Nox2 knockout cells under starvation was further confirmed by immunoblotting (Fig. 6C).

Discussion

The abilities of EC to proliferate, to be quiescent in monolayer, and to undergo apoptosis during remodeling are important determinants relating to angiogenesis, wound healing, and many diseases, including atherosclerosis. Nutrient deprivation-induced oxidative stress and EC injury occur during ischemia, angioplasty, and organ transplantation. Although regeneration of the endothelium might represent a potential therapeutic strategy for diseases with endothelial injuries [23], the mechanisms involved in the regulation of EC growth and apoptosis are far from clear. The present study revealed, for the first time, a critical role for Nox2 and its product, ROS, in nutrient-deprivation-induced EC cell cycle withdrawal and apoptosis.

There are at least three Noxes (Nox1, Nox2, and Nox4) reported to be expressed in EC [3,13]. We found that Nox1 mRNA expression was hardly detectable in proliferating EC and was below the level of detection after starvation. Our results were in accordance with previous reports that Nox1 seems not to be important in EC ROS generation under basal conditions because depletion of Nox1 with siRNA did not alter basal EC ROS generation [24].

With more than one Nox isoform expressed simultaneously in EC, it was possible that Nox2 and Nox4 might have different roles in EC function. An important finding from the present study is that the expression and activity of Nox2 (but not Nox4) are closely coupled with the expression and activity of the cyclin-dependent kinase inhibitor, p21^{cip1} (and possibly p53 as well), and involved in EC cell cycle regulation. When EC were maintained in complete growth medium and engaged actively in the cell cycle progression, the expression of both Nox2 and p21^{cip1} was low. However, when EC were subjected to nutrient deprivation for

more than 24 h, Nox2 expression was robustly elevated and this was accompanied by: (i) a significant increase in ROS production, (ii) dramatic inductions of p21^{cip1} and p53, and (iii) a significant increase in EC apoptosis.

The CDKI p21^{cip1} has been reported to regulate cell cycle progression and inhibit apoptosis of mature EC in a concentration-dependent manner [6]. At low levels, p21^{cip1} promotes cell cycle progression, whereas at high concentrations, it leads to cell cycle arrest. Low levels of p21^{cip1} also were found to be necessary to prevent EC from undergoing apoptosis [6]. The tumor suppressor gene product p53 plays an essential role in the signaling pathways of EC apoptosis and senescence through its ability to activate transcriptionally a wide variety of target genes (including p21^{cip1}) that are involved in cell cycle arrest and apoptosis [8,25]. Both p21^{cip1} and p53 have been reported to be redox-sensitive [8,25]. We have observed here that starvation-induced accumulation of p21^{cip1} and p53 requires an increase in Nox2-derived ROS. There was a specific relationship between the expression and the activity of Nox2 and p21^{cip1} as revealed by our time-course experiments. This relationship was further confirmed by in vitro depletion of Nox2. With very little Nox2 protein available, cells failed to trigger a significant ROS response to nutrient deprivation and subsequently failed to accumulate p21^{cip1} and p53 to promote apoptosis. The important role of Nox2 in redox regulation of p21^{cip1} was highlighted by experiments using CMEC isolated from Nox2 knockout mice. In the absence of Nox2, although Nox4 expression was significantly increased 4.8±0.5-fold after 36 h of starvation, there was no significant increase in O₂^{•-} production and p21^{cip1} expression. More importantly, Nox2 knockout CMEC were resistant to starvation-induced cell apoptosis.

Oxidative stress has multiple effects on cell function depending on the amount and subcellular location of ROS generated. In EC, Nox2 was detected mainly in the perinuclear region in association with the cytoskeleton [19]. The particular perinuclear location of Nox2, in principle, might facilitate the interaction of ROS specifically with certain cell cycle signaling molecules, such as p21^{cip1} and p53. This was supported by our DCF fluorescence experiments showing that tiron-inhibitable ROS production was mainly detected around the nuclear region (Fig. 3B). In general, the key features of endothelial Nox2 oxidase activation are similar to the phagocytic oxidase that requires the presence of regulatory subunits, such as p47^{phox} [10,20]. We also found that the expression of p47^{phox} was increased in a manner similar to the expression of Nox2 in response to nutrient deprivation.

At the molecular level, Nox4 is only distantly related to Nox2, exhibiting only a 39% amino acid sequence homology. Nox4 does not seem to exhibit a site linked to the binding of p47^{phox} [26], and the known regulatory subunits (p67^{phox} or rac1) are not required for Nox4 activity [21]. Therefore, Nox4 has been suggested to be a constitutively activated enzyme or it could have another specific mechanism of regulation. We found that Nox4 is abundantly expressed in HMEC1 under conditions of both cellular proliferation and growth arrest. Although serum removal caused an increase in Nox4 expression, the time and scale of Nox4 elevation did not match the actual increase in the level of ROS production seen at 36 h of starvation. Furthermore, the Nox4 response to starvation was a moderate and slow process, which seemed not to be critical for the robust increases in ROS production seen at 36 h. In addition, in Nox2 knockout CMEC, although Nox4 expression was increased approximately fourfold at 36 h of starvation, there was no significant increase in ROS production, nor was there a significant induction of p21^{cip1} or cell apoptosis. It is also worth noting that there was a notable delay in the G₁- to S-phase transition observed in Nox2 knockout CMEC compared to wild-type cells. A possible explanation for this is that a low level of Nox2 might be necessary to promote or synergize with Nox4 activity for the optimum ROS level required for full EC cell cycle progression and proliferation. This could also provide an explanation for previous observations that Nox2-deficient mice displayed decreased

neointimal formation after arterial injury [7], and Nox2^{-/-} EC had much less of a proliferative response during 24 h of static culture [22].

In conclusion, we report for the first time that Nox2 has a specific role in EC function. Nox2-derived ROS, by participating in the regulation of p21^{cip1} and p53, are involved in the control of EC cell cycle arrest and apoptosis.

Acknowledgments

This work is supported by a BBSRC grant (BB/D009510/1) and a Wellcome Trust grant (078637/Z/05/Z) to J.-M. Li.

Abbreviations

ROS	reactive oxygen species
EC	endothelial cells
HMEC1	human dermal microvascular EC
CMEC	coronary microvascular EC

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Fig. 1.

Time course of cell proliferation, apoptosis, and the levels of ROS production in HMEC1 under starvation (0.2% FCS medium). (A) Cell proliferation was assessed by cell counting. The proliferation index at each time point was calculated as fold changes relative to the cell number at 0 h (Index 1). (B) Cell apoptosis was detected by propidium iodide flow cytometry. (C) NADPH-dependent $O_2^{\bullet-}$ production was measured by lucigenin chemiluminescence. RLU, relative light unit. (D) Enzyme inhibitor effects on $O_2^{\bullet-}$ production after 36 h of starvation. * $p < 0.05$ for indicated value versus the value at 0 h. † $p < 0.05$ for indicated value versus the control value. $n = 3$ separate experiments.

Fig. 2. Quantitative real-time PCR analysis of expression of mRNA for Nox1, Nox2, Nox4, p47^{phox}, and p21^{cip1} during a time course of starvation. HMEC1 starved in 0.2% FCS medium were harvested at 0, 12, 24, and 36 h. The results were expressed as molar/molar ratio relative to the level of mRNA expression of β -actin in the same sample. * $p < 0.05$; significant increase for the indicated value versus the value at 0 h. $n = 3$ separate cell cultures.

Fig. 3. Changes in protein expression for Nox2, Nox4, p21^{cip1}, and p53; ROS production; and cell cycle analysis during proliferation (growth medium) versus starvation (0.2% FCS medium with or without apocynin). (A) Immunoblotting for the protein expression of Nox2, Nox4, p47^{phox}, p21^{cip1}, and p53. A neutrophil membrane preparation was used as a positive control for the detection of Nox2, and cell lysate of HEK2 cells was used as positive control for the detection of Nox4. The protein expression of α-tubulin in the same samples was used as a loading control. (B) *In situ* ROS production. Cells were cultured on chamber slides and ROS production was detected by DCF fluorescence and quantified digitally by confocal microscopy. Tiron was used to confirm the detection of O₂^{•-}. (C) Cell cycle analysis by propidium iodide flow cytometry. **p*<0.05; value in 0.2% FCS versus value in growth medium. †*p*<0.05; significant decrease for the value with tiron versus the value without tiron. *n*=3 separate cell cultures.

Fig. 4.

In vitro depletion of Nox2 for the expression of p21^{cip1} and p53, the level of O₂^{•-} production, and cell apoptosis. HMEC1 were transfected with empty vector (pcDNA3.1) as control or with a full-length Nox2 antisense cDNA inserted into the same vector. (A) Immunoblotting of Nox2, p21^{cip1}, and p53. α-Tubulin was used as a loading control. (B) O₂^{•-} production was measured by lucigenin (5 μM) chemiluminescence. NADPH was added after 20 min of reading and tiron was added after 40 min of reading. RLU, relative light units. (C) The number of cells that had undergone death was counted by trypan blue exclusion. **p*<0.05; significant increase for value in 0.2% FCS versus value in growth medium. *n*=3 separate cell transfections.

Fig. 5. mRNA expression of Nox2, Nox4, and p21^{cip1}; ROS production and cell cycle analysis in CMEC in growth medium or in 0.2% FCS medium for 36 h. (A) mRNA expression of Nox4, Nox2, and p21^{cip1} expressed as molar/molar ratio to the level of β -actin detected in the same sample. (B) NADPH-dependent O₂⁻ production was measured by lucigenin chemiluminescence. RLU: relative light unit. (C) Cell cycle analysis by propidium iodide flow cytometry. * $p < 0.05$ significant increase for the values in 0.2% FCS versus values in growth medium. $n = 3$ separate CMEC isolations (6 mice/per group/per isolation).

Fig. 6.

Subcellular localization; protein expression of Nox2, p21^{cip1}, and p53; and cell apoptosis in CMEC. (A) Confocal microscopic images of wild-type CMEC. Normal goat and rabbit IgG (5 µg/ml) were used as background controls for Nox2 and p21^{cip1}, respectively. (B) Confocal microscopic images of wild-type and Nox2 knockout CMEC. Cells were double stained for p53 (FITC, green fluorescence) and propidium iodide (red fluorescence). Arrow indicates nuclear fragmentation surrounded by apoptotic bodies. Scale bar, 20 µm. (C) Immunoblotting for the differences in p53 protein expression between wild-type and Nox2 knockout CMEC after 36 h of serum starvation. α-tubulin was used as a loading control.