Endothelial Cells Negatively Modulate Reactive Oxygen Species Generation in Vascular Smooth Muscle Cells
Role of Thioredoxin

Shaoping Xu, Ying He, Martina Vokurkova, Rhian M. Touyz

Abstract—In intact vessels, endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) act as an integrated system, possibly through reactive oxygen species (ROS). Using a coculture system we tested whether ECs modulate VSMC redox status by regulating activity of NAD(P)H oxidase and antioxidants. VSMC production of O$_2^•$-, H$_2$O$_2$, and NO was assessed using fluoroprobès and amplex-red. NAD(P)H oxidase subunit expression and oxidase activity were determined by Western blotting and chemiluminescence, respectively. Expression of thioredoxin, SOD, growth signaling pathways (PCNA, p21cip1, CDK4, ERK1/2, p38MAPK) was evaluated by immunoblotting. Thioredoxin activity was assessed by the insulin disulfide reduction assay. In cocultured conditions, VSMC ROS production was reduced by $\approx 50\%$ without changes in NAD(P)H oxidase expression/activity versus monoculture ($P<0.05$). This was associated with decreased cell growth ($P<0.05$). Expression of Cu/Zn SOD and thioredoxin was increased in coculture versus monoculture VSMCs ($P<0.01$). Pretreatment of ECs with L-NAME (NOS inhibitor), NS-398 (Cox2 inhibitor), and HET0016 (20-HETE inhibitor) did not influence VSMC ROS formation, whereas CDNB, thioredoxin reductase inhibitor, abolished ROS modulating effects of ECs. These findings indicate that in a coculture system recapitulating intact vessels, ECs negatively regulate ROS production in VSMCs through thioredoxin upregulation. Functionally this is associated with growth inhibition. The modulatory actions of ECs are independent of NOS/NO, Cox2, and HETE and do not involve NAD(P)H oxidase. Our data identify novel mechanisms whereby ECs protect against VSMC oxidative stress, a process that may be important in maintaining vascular integrity. (Hypertension. 2009;54:00-00.)

Key Words: coculture ■ oxidative stress ■ vascular cells ■ antioxidants ■ signal transduction

Interactions between endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) are fundamental in maintaining vascular structure and function. ECs and VSMCs act as a coupled system for transmission of signals from the endothelium to the underlying vascular media and vice versa. Communications between ECs and VSMCs occur through synthesis and release of mediators or through direct cell-to-cell contact. ECs regulate vascular tone through vasorelaxing molecules such as nitric oxide (NO), hydroxyeicosatetraenoic acid (HETE), and prostacyclin (PGI). Recent studies using a coculture system to mimic in vivo relations between ECs and VSMCs demonstrate that ECs regulate VSMC proliferation, migration, differentiation, and gene expression. All of these cellular processes are regulated by reactive oxygen species (ROS). However, whether ECs influence redox status in VSMCs is unclear, because most studies examining the role of ROS in VSMCs have been performed in monocultures.

All vascular cell types, including ECs, VSMCs, and adventitial fibroblasts produce superoxide (O$_2^•$-), hydrogen peroxide (H$_2$O$_2$), and NO. Under physiological conditions, ROS are produced in a controlled manner at low concentrations and function as inter- and intracellular signaling molecules. In pathological conditions, increased activity/expression of ROS generating enzymes or decreased defenses by antioxidants in the vasculature result in increased bioavailability of ROS, leading to oxidative stress and vascular damage.

Among ROS-generating enzymes, NAD(P)H oxidases are particularly important. All subunits of the prototype neutrophil NAD(P)H oxidase, including gp91phox (now termed Nox2), p22phox, p47phox, p67phox, and the small G protein Rac1, as well as Nox2 isoforms Nox1, Nox4, and NoxA5 are expressed in human vascular cells and involved in O$_2^•$- generation. H$_2$O$_2$ is produced primarily from dismutation of O$_2^•$- by superoxide dismutase (SOD) (cytosolic SOD [Cu/Zn-SOD], mitochondrial SOD [Mn-SOD], and extracellular SOD [EC-SOD]) and is scavenged by peroxidases, among which peroxiredoxin is particularly important. The peroxiredoxin system comprises thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH. It is abundant in VSMCs and has high affinity for H$_2$O$_2$. Thioredoxin is regulated by vitamin D3 upregulated protein 1

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(VDUP-1), an endogenous inhibitor, which when overexpressed significantly reduces thioredoxin activity.23

In pathological conditions VSMC-derived O2•− has been implicated in the quenching of NO in ECs to form injurious ONOO−.24,25 To our knowledge this interaction has not been demonstrated directly, and there is little evidence to show that under basal conditions there is cross-talk between ECs and VSMCs with respect to ROS production. In the present study, using a cocultured system to recapitulate in vivo conditions,26 we tested the hypothesis that ECs modulate redox state and cell growth in VSMCs. We also investigated putative mechanisms whereby ECs influence ROS generation in VSMC, focusing specifically on NAD(P)H oxidase and the antioxidant enzymes SOD and thioredoxin.

Materials and Methods

Cell Culture

Vascular smooth muscle cell were isolated from resistance arteries obtained from gluteal biopsies of subcutaneous tissue from healthy volunteers (age 30 to 65 years), cultured in Dulbecco Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) as we previously described.13 Identity of VSMCs was verified by positive immunostaining with anti–α-smooth muscle actin antibody, anti-smooth muscle myosin antibody, and anticalponin antibody and negative immunostaining with anti-human fibroblast surface protein and anti–von Willebrand factor antibody (data not shown). Human microvascular ECs were purchased from Cascade Biologies/Invitrogen (Portland, Ore) and maintained in Medium-131 with endothelial cell supplements.

Coculture System

Endothelial cells were plated in 6-well cell culture companion plates (=8×10^5 cells per well; BD Falcon cell culture insert companion plates and insert, BD Bioscience) and grown for 24 hours before coculture. Vascular smooth muscle cells (passages 5 to 8; =1×10^5 cells per insert) were then plated onto the insert membrane, a 13-μm-thick porous polyethylene terephthalate membrane with 0.4-μm pores configured at a density of 1.6×10^6 pores/cm². Both cell types were maintained in their specific culture medium for 24 hours. Thereafter both cell types were placed in DMEM containing 2%-containing FBS for another 48 hours. Previous experiments showed that 2% FBS promotes submaximal cell proliferation but does not itself induce their differentiation. For control experiments, VSMCs were plated as a monoculture onto the insert in the absence of ECs and exposed to DMEM/2% FBS as for the coculture setup.

Protocols

In some experiments, ECs were exposed to vehicle, eNOS inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME; 1 mmol/L), Cox2 inhibitor, N-2-cyclohexyloxy-4-nitrophenyl-methanesulfonamide (CDNB; 0.15 mol/L), or 20-HETE inhibitor, N-hydroxy-N-(4-buty1-2-methyl-phenyl)-formamidine (HET0016; 1 mol/L). Concen-
trations of pharmacological inhibitors used have been shown to be effective and specific.27–29

Measurements of Intracellular O2•−, H2O2, and NO

Mono- and cocultured VSMCs were harvested with trypsin. Trypsin (1 mL) was added to the insert or well for 2 to 3 minutes to initiate detachment. PBS +10% FBS was then added, and cells were washed by centrifugation and resuspended in PBS (250 μL). Cells were incubated in the dark (30 minutes, 37°C) with dihydroethidium (DHE; 5 μmol/L), to evaluate O2•− levels, dichlorofluorescein diacetate (DCF-DA; 4 μmol/L), to evaluate H2O2, or trypan blue (or 4,5-diaminofluorescein diacetate (DAF-FM diacetate;4 μmol/L), to evaluate NO levels. Flow cytometry was used to select a homogeneous population of 10,000 live cells according to forward and side scatter.

Determination of Extracellular H2O2

Culture media were changed to phenol-free medium for the last 24 hours of coculture and collected for extracellular H2O2 assay using the amplex red hydrogen Peroxide/Peroxidase Assay Kit (A22188, Molecular Probes) according to the manufacturer’s instructions. Western Blotting

Cocultured and monocultured VSMCs were lysed. Total protein (30 μg) was separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with primary antibodies for Nox4 (rabbit polyclonal, Santa Cruz), Nox2/gp91phox (rabbit polyclonal, gift from M. Quinn, Montana State University, Bozeman), p47phox (rabbit polyclonal, gift from M. Quinn), Rac1 (mouse monoclonal, Santa Cruz), Cu/Zn SOD (rabbit polyclonal, Bioversion), thioredoxin (mouse monoclonal, Abcam), Vitamin D3 upregulated protein-1 (VDUP-1, rabbit polyclonal, Zymed Laboratories), ERK1/2 and phospho-ERK1/2 (rabbit polyclonal, Cell Signaling), p38MAPK and phospho-p38MAPK, (rabbit polyclonal, Cell Signaling), proliferating cell nuclear antigen (PCNA; mouse monoclonal, Santa Cruz), cyclin-dependent kinase 4 (CDK4; mouse monoclonal, Santa Cruz), and p21cip1 (rabbit polyclonal, Santa Cruz). Immunoreactive bands were visualized using HRP-conjugated antirabbit or antimouse IgG (Sigma) and ECL Western blot analysis system (Pierce). Optical density was quantified by ImageQuant Software (Molecular Dynamics) and expressed as percentage of control.

Thioredoxin Activity Assay

The modified insulin disulfide reduction assay was performed to measure thioredoxin activity as described.30,31

Statistical Analysis

Experiments were repeated 3 to 6 times in duplicate or triplicate. Data are expressed as mean±SEM and analyzed by ANOVA or by unpaired Student t test as appropriate. P<0.05 was considered significant.

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Results

ECs Decrease ROS Production in VSMCs

In VSMCs cocultured with ECs, generation of intracellular O2•− and H2O2 was significantly decreased compared with monocultured VSMCs (absence of ECs; Figure 1A and 1B). Levels of NO in VSMCs were unchanged by ECs (data not shown). Because H2O2 is cell membrane–permeable, we also measured extracellular H2O2 levels to support our intracellular ROS measurements. As shown in Figure 1C, extracellular H2O2 levels were also decreased in cocultured VSMC compared to monocultured VSMC. This effect appears to be independent of endothelial-derived NO, because VSMC ROS levels were still significantly reduced even when ECs were pretreated with the eNOS inhibitor L-NAME (Figure 1C). To address whether cyclooxygenase2/prostacyclin (Cox2/PGI2) or 20-HETE mediated this modulatory effect, ECs were incubated NS-398 or HET0016, selective Cox2, and 20-HETE inhibitors, respectively. H2O2 generation in cocultured VSMCs was not significantly modified by either inhibitor (Figure 1C), suggesting that, at least in this coculture system, Cox2/PGI2, and 20HETE are not involved in the ROS modulatory effects of ECs in cocultured VSMCs. NAD(P)H Oxidase Expression and Activity in VSMCs Are Not Altered by ECs

Because NAD(P)H oxidase is the major source of ROS in vascular cells, we next assessed the expression of NAD(P)H...
oxidase subunits Nox2/gp91phox and Nox4 (membrane binding and catalytic subunits) and p47phox and small G protein Rac1 (cytosolic subunits) in cocultured VSMC. Although there was some modulation of NAD(P)H oxidase subunit expression in VSMCs when cocultured with ECs, effects were not significant (Figures 2A and 2B and S1, please see http://hyper.ahajournals.org).

To evaluate in further detail whether ECs influence the function of NAD(P)H oxidase in VSMCs, activity of the oxidase by lucigenin chemiluminescence was measured. As shown in Figure 2C, activation of NAD(P)H oxidase is similar in VSMCs grown in monoculture and in coculture. Taken together these data suggest that NAD(P)H oxidase is probably not involved in the modulation of ROS levels in VSMCs by ECs.

**ECs Upregulate SOD and Thioredoxin in Cocultured VSMCs**

To determine whether antioxidant systems in VSMCs are modified by ECs, expression of Cu/Zn SOD and thioredoxin in coculture VSMCs compared to monoculture VSMCs.

**ECs Influence H₂O₂ Formation in VSMCs Through Thioredoxin**

We further investigated the putative role of the thioredoxin system by evaluating thioredoxin activity in VSMCs using the insulin disulfide reduction assay. As shown in Figure 4A, EC coculture increased VSMC activity of thioredoxin. To assess whether thioredoxin inhibition influences EC modulatory effects on VSMC ROS production, H₂O₂ was measured in coculture VSMCs in which VSMCs were exposed to CDNB. As demonstrated in Figure 4B, CDNB inhibited the modulatory action of ECs on VSMC production of H₂O₂.

**Expression of Vitamin D3 Upregulated Protein-1 in HVSMCs**

VDUP-1 content was determined in VSMCs by Western blot. As shown in Figure 4C, VDUP-1 expression was similar in VSMCs grown in monoculture and in coculture.

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**Figure 1.** Decreased generation of reactive oxygen species in VSMCs grown in coculture (CC) with ECs compared with VSMCs grown as monoculture (MC). A and B, VSMCs were cocultured with ECs for 72 hours and harvested with trypsin. Cells were exposed for 30 minutes to 5 μmol/L DHE or 5 μmol/L DCFDA, and fluorescence was measured by flow cytometry. DHE fluorescence for intracellular O₂⁻ (A), and DCFDA fluorescence for H₂O₂ (B). C, VSMCs were cocultured with ECs for 72 hours. ECs were treated with either vehicle or L-NAME (1 mmol/L), NS-398 (10 μmol/L), or HET0016 (1 μmol/L) during the last 48 hours of CC. Media was collected from the VSMC compartment and extracellular H₂O₂ levels determined by amplex red fluorescence. Data are expressed as percentage of control (MC) with control taken as 100%. Results are mean±SEM of 6 independent experiments. *P<0.05, **P<0.01, compared with MC.

**Figure 2.** Expression of NAD(P)H oxidase subunits, Nox4 (A), and p47phox (B) in monoculture (MC) and coculture (CC) VSMCs. VSMCs were cocultured with ECs for 72 hours and were analyzed by immunoblotting with antibodies recognizing human Nox4 and p47phox. C, Activation of NAD(P)H in VSMCs grown as MC and CC as assessed by lucigenin chemiluminescence. Data are expressed as percentage of control (MC) with control taken as 100%. Results are mean±SEM of 6 independent experiments. RLU indicates relative light units.
Effect of ECs on VSMC Growth

To evaluate the functional significance of EC:VSMC interaction, indices of cell growth were determined, specifically expression of PCNA and cell cycle proteins p21cip1 (inhibitor) and CDK4 (promoter). As demonstrated in Figure 5, expression of PCNA and p21cip1 was decreased and increased, respectively, in cocultured VSMCs versus monocultured VSMCs. CDK4 expression was not significantly altered. Activation of ERK1/2 and p38MAPK signaling molecules important in cell growth was also evaluated. Endothelial cells did not significantly modulate phosphorylation of ERK1/2 and p38MAPK in coculture versus monoculture VSMCs \(P > 0.05\), and had no effect on total concentration of these proteins (data not shown).

Discussion

Communication between ECs and VSMCs impacts vascular function and structure by influencing contractile, mitogenic, migratory, and inflammatory properties, all of which are regulated, to varying degrees, by ROS. The capability of different cell types to control each other’s activities led us to question how ECs modulate VSMCs, specifically with respect to ROS generation. Using a human cocultured system as a model to understand interactions between ECs and VSMCs, we demonstrate the novel findings that under basal conditions, microvascular ECs negatively modulate VSMC levels of \(O_2^{\bullet} \) and \(H_2O_2\) and attenuates cell growth in VSMCs compared with monoculture VSMCs. Mechanisms underlying our findings involve upregulation of antioxidant enzymes Cu/Zn SOD and thioredoxin and appear to be independent of NAD(P)H oxidase. The EC-derived factor(s) responsible for these actions remain unclear, although eNOS, Cox2, and HETE do not seem to play a major role. Functionally, ECs temper VSMC growth, a process that is not associated with changes in ERK1/2 and p38MAPK. Taken together, our data suggest that under basal conditions, ECs protect against VSMC oxidative stress by upregulating antioxidant systems, specifically thioredoxin. Such events may be important in the physiological regulation of cell growth. In intact vessels, negative modulation by the endothelium may play a role in maintaining vascular smooth muscle integrity and preventing against oxidative injury.

Reactive oxygen species are critically involved in signal transduction and the physiological regulation of vascular...
function. In pathological conditions, increased bioavailability of ROS (oxidative stress) triggers signaling events that promote endothelial dysfunction, vascular inflammation, and arterial remodeling. All vascular cell types produce ROS. Vascular ROS-generating systems, cellular ROS homeostasis, and redox-dependent signaling pathways have been characterized mainly in monocultures. However, in the intact vessel, the endothelium is in close proximity with the underlying vascular smooth muscle, and there is dynamic interaction between these cell layers through cell-cell coupling and through secreted mediators. In the present in vitro study, in vivo conditions were recapitulated by examining the redox system in VSMCs that were in contact with ECs. We show that production of $O_2^-$ and $H_2O_2$ by VSMCs is reduced in the presence of ECs. Our results support in vivo studies demonstrating that the endothelium regulates vascular tone partly by modulating ROS production in VSMCs. Because of the technical challenges to accurately measure $O_2^-$, $H_2O_2$, and NO levels, we used multiple methods to assess ROS status, including lucigenin chemiluminescence, DHE fluorescence, DCFDA fluorescence, DAF fluorescence, and the amplex red assay. All of these methods have some limitations, as recently detailed by Cai et al. However, independent of the method used, our results all changed in the same direction, indicating that the data represent true alterations in the different experimental conditions.

Mechanisms whereby ECs influence VSMC ROS formation are unclear, but eNOS-derived NO may be important. Nitric oxide is a potent vasodilator and inhibits VSMC proliferation and migration, platelet aggregation, and monocyte adhesion, in part through ROS. ROS generation in VSMCs cocultured with L-NAME-treated ECs was similar to that in VSMCs cocultured with vehicle-treated ECs, indicating that negative modulatory effects of ECs are probably eNOS-independent. However, because NO has a very short half-life and diffuses only short distances from its site of production, we cannot exclude the possibility that NO may be converted to nitrate and nitrite before it takes effect in VSMCs. We also explored the possibility that Cox2-derived PGi2 and 20-HETE, other important EC-derived mediators, may influence redox homeostasis in VSMCs. However, $H_2O_2$ levels remained low in VSMCs cocultured with NS-398 and HET0016, suggesting that these systems are not involved in the regulatory ROS actions in VSMCs by ECs.

A major source of vascular ROS is Nox-based NAD(P)H oxidase, which when activated leads to increased $O_2^-$ generation. Basal Nox activity is low and contributes to ROS involved in cell signaling. In our study NAD(P)H oxidase activity, as assessed by chemiluminescence and expression of NAD(P)H oxidase subunits Nox2, Nox4, p47phox, and Rac1, were similar in VSMCs in monoculture and in coculture, even though ROS production was reduced in cocultured cells. This dissociation between activity of the oxidase and ROS formation suggests that ECs do not significantly regulate NAD(P)H oxidase in VSMCs, at least in the basal unstimulated state.

ROS homeostasis is determined by both $O_2^-$-generating enzymes and by antioxidants. Because VSMC NAD(P)H oxidase was not significantly altered by ECs we probed the possibility that decreased ROS in cocultured VSMCs is related to upregulation of antioxidant systems. Expression of Cu/Zn SOD and thioredoxin, major vascular antioxidant enzymes, was increased in cocultured VSMCs compared with monoculture VSMCs, implicating an EC-derived factor in this phenomenon. Our findings are supported by others which demonstrated that manganese SOD expression in VSMCs is upregulated via EC-dependent pathways. Because SOD dismutates $O_2^-$ to $H_2O_2$, it would be predicted that in the presence of increased SOD, $H_2O_2$ formation should be increased. However, we found that levels of both intracellular and extracellular $H_2O_2$ were reduced in coculture, in spite of increased SOD content, and hence tested whether thioredoxin may be the antioxidant responsible for decreased ROS in these conditions. In line with the expression pattern of thioredoxin, activation of thioredoxin reductase was increased. To further support a role for the thioredoxin system in the modulation of VSMC ROS generation by ECs, we demonstrated that vascular thioredoxin reductase inhibition by CDNB abolished EC-induced actions. These phenomena were unrelated to changes in VDUP-1, the endogenous thioredoxin inhibitor, because VDUP-1 was unperturbed in VSMCs by EC coculture. Taken together our data suggest that ECs negatively regulate ROS bioavailability in VSMCs by influencing the thioredoxin system, through mechanisms that do not involve VDUP-1.
To address the functional consequences of EC-VSMC interaction, growth responses in VSMCs were evaluated as assessed by PCNA content, cell cycle regulators, and growth signaling molecules. PCNA is a sensitive molecular marker of cell proliferation. Molecular processes associated with cell growth were attenuated in cocultured VSMCs compared with monocultured cells, an effect associated with increased expression of the cell cycle inhibitor, p21cip1. These events appear to be independent of ERK1/2 and p38MAPK, because phosphorylation of these kinases in VSMCs was unaffected by ECs. Because MAP kinases are redox-sensitive, it is possible that in stimulated conditions or in vascular injury where oxidative stress is increased, activation of MAP kinases may be important. This is evidenced by the myriad studies examining monocultures, where Ang II, ET-1, aldosterone, cytokines, and other vasoactive agents have been shown to activate ERK1/2 and p38MAPK in a redox-sensitive manner.44–46

In conclusion, examining a human cocultured system as a model to understand cross-talk between ECs and VSMCs, we provide novel data to show that under basal conditions, ECs negatively modulate VSMC production of ROS, an effect associated with attenuated cell growth. Mechanisms underlying this phenomenon involve upregulation of thioredoxin through, as yet, unknown EC-derived factors. Endothelial cell L-NAME, Cox2, and HETE do not seem to be critically involved. Endothelial cells seem to protect against VSMC oxidative stress by upregulating antioxidant systems, specifically thioredoxin. Such processes may be important in the physiological regulation of VSMC function.

**Perspectives**

Endothelial and vascular smooth muscle cells act as a coupled system for transmission of signals from the endothelium to the underlying vascular media to control vascular function and structure. Mechanisms for this are complex. Here we described a novel regulatory role of the endothelium, whereby ECs negatively regulate ROS production in VSMCs through thioredoxin upregulation, an effect associated with attenuated cell growth. These findings highlight the important cross-talk between ECs and VSMCs and suggest that in basal conditions, endothelial cells have an apparent protective effect by dampening ROS generation and cell growth in VSMCs. In intact vessels in physiological conditions, such responses may prevent oxidative injury. Endothelial cell–derived factors responsible for EC-mediated ROS actions in VSMCs remain unclear but are currently under investigation using proteomic strategies. In pathological conditions where endothelial function is impaired the protective actions observed in our study may be altered resulting in vascular damage. Future investigations will address this at the molecular level using cocultures in which endothelial cells are stressed by stretch and cytokines.

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**Disclosures**

None.

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SUPPLEMENTARY DATA

Endothelial Cells Negatively Modulate Reactive Oxygen Species Generation in Vascular Smooth Muscle Cells: Role of Thioredoxin

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Abstract

In intact vessels, endothelial cells (EC) and vascular smooth muscle cells (VSMC) act as an integrated system, possibly through reactive oxygen species (ROS). Using a co-culture system we tested whether ECs modulate VSMC redox status by regulating activity of NADPH oxidase and antioxidants. VSMC production of \( \bullet O_2^- \), \( H_2O_2 \) and NO was assessed using fluoroprobes and amplex-red. NADPH oxidase subunit expression and oxidase activity were determined by western blotting and chemiluminescence respectively. Expression of thioredoxin, SOD, growth signaling pathways (PCNA, p21cip1, CDK4, ERK1/2, p38MAPK) was evaluated by immunoblotting. Thioredoxin activity was assessed by the insulin disulfide reduction assay. In cocultured conditions, VSMC ROS production was reduced by \( \sim 50\% \) without changes in NADPH oxidase expression/activity versus monoculture (p<0.05). This was associated with decreased cell growth (p<0.05). Expression of Cu/Zn SOD and thioredoxin was increased in coculture versus monoculture VSMCs (p<0.01). Pretreatment of ECs with L-NAME (NOS inhibitor), NS-398 (Cox2 inhibitor) and HET0016 (20-HETE inhibitor) did not influence VSMC ROS formation, whereas CDNB, thioredoxin reductase inhibitor, abolished ROS modulating effects of ECs. These findings indicate that in a coculture system recapitulating intact vessels, ECs negatively regulate ROS production in VSMCs through thioredoxin upregulation. Functionally this is associated with growth inhibition. The modulatory actions of ECs are independent of NOS/NO, Cox2 and HETE and do not involve NADPH oxidase. Our data identify novel mechanisms whereby ECs protect against VSMC oxidative stress, a process that may be important in maintaining vascular integrity.

Materials and Methods

Cell Culture

Vascular smooth muscle cell were isolated from resistance arteries obtained from gluteal biopsies of subcutaneous tissue from healthy volunteers (age 30 to 65 years), cultured in Dulbecco Modifies Eagle’s Medium (DMEM) with 10% fetal bovine serum (FBS) as we previously described (13). Identity of VSMCs was verified by positive immunostaining with anti-\( \alpha \)-smooth muscle actin antibody, anti-smooth muscle myosin antibody, and anti-calponin antibody and negative immunostaining with anti-human fibroblast surface protein and anti-Von Willebrand factor antibody (data not shown). Human microvascular endothelial cell (EC) were purchased from Cascade Biologics/Invitrogen (Portland, USA) and maintained in Medium-131 with endothelial cell supplements.

Coculture system

Endothelial cells were plated in 6-well cell culture companion plates (~ 8x10^5 cells/well) (BD Falcon cell culture insert companion plates and inserts (BD Bioscience, San Jose, CA)) and grown for 24 hours prior to coculture. Vascular smooth muscle cells (passages 5-8) (~ 1x10^5 cells/insert) were then plated onto the insert membrane, a 13-\( \mu \)-m-thick porous polyethylene terephthalate membrane with 0.4-\( \mu \)-m pores configured at a density of 1.6x10^6 pores/cm^2. Both cell types were maintained in their specific culture medium for 24 hours. The culture medium was then changed to 2% -containing FBS for another
48 hours. For control experiments, VSMCs were plated as a monoculture onto the insert in the absence of ECs.

**Protocols**

In some experiments, ECs were exposed to vehicle, eNOS inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME) (1 mM), Cox 2 inhibitor, N-2-cyclohexyloxy-4-nitrophenyl-methanesulfonamide (NS-398) (10μM) or 20-HETE inhibitor, N-hydroxy-N'-(4-butyl-2-methyl-phenyl)-formamidine (HET0016) (1 μM) during the last 48 hours of coculture. In some experiments VSMCs were treated with dinitrochlorobenzene (CDNB) (0.15μM). Concentrations of pharmacological inhibitors used have been shown to be effective and specific (27-29).

**Measurements of intracellular •O₂⁻, H₂O₂ and NO**

Mono- and co-cultured VSMCs were harvested with trypsin, resuspended in PBS containing 2% FBS, and filtered through a nylon mesh. Cells were then incubated in the dark (30 minutes, 37°C) with dihydroethidium (DHE) (5 μM), which is oxidized to ethidium by •O₂⁻, dichlorofluorescein diacetate (DCF-DA) (4 μM), which is oxidized to dichlorofluorescein (DCF) by H₂O₂ and peroxidases, or to 4,5-diaminofluorescein-diacetate (DAF-FM diacetate) (4 μM), which reacts with intracellular NO to form a fluorescent heterocycle. Flow cytometry (FACScan, Becton Dickinson, Mississauga, ON) was used to select a homogeneous population of 10,000 live cells according to forward and side scatter. The geometric mean of ethidium (excitation 488nm, emission 610nm), DCF (488/530nm excitation/emission) or DAF (495/515nm excitation/emission) fluorescence intensity (arbitrary units per cell) in the population was used for analysis. Results are expressed as the percentage relative to control.

**Determination of Extracellular H₂O₂**

Culture media were changed to phenol-free medium for the last 24-hour of coculture and collected for extracellular H₂O₂ assay using the amplex red hydrogen Peroxide/Peroxidase Assay Kit (A22188, Molecular Probes, Eugene, Oregon) according to the manufacture’s instructions. Briefly, 50μl of culture medium and 50μl of working solution of 100μM amplex red reagent and 0.2U/ml horseradish peroxidase (HRP) were mixed and incubated in the dark at room temperature for 30 minutes. In the presence of peroxidase, the amplex red reagent reacts with H₂O₂ to produce the red-fluorescent oxidation product resorufin. Fluorescence was then measured with a fluorescence microplate reader using excitation at 540nm and emission at 590nm. Concentration of H₂O₂ from each sample was calculated according to a H₂O₂ standard curve and expressed as the percentage of control.

**Western blotting**

Cocultured and mono-cultured VSMCs were lysed in 0.5% Triton X-100 lysis buffer containing protease inhibitors (2mM Na₃VO₄, 1mM PMSF, 1μg/ml Leupeptin, 1μg/ml Aprotinin, and 1μg/ml Pepstatin). Total protein (30 μg) was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with either 10% or 12% acrylamide depending on the size of the protein of interest, transferred to nitrocellulose membrane, and probed with primary antibodies for Nox4 (rabbit polyclonal),
Nox2/gp91phox (rabbit polyclonal), p47phox (rabbit polyclonal), Rac1 (mouse monoclonal), Cu/Zn SOD (rabbit polyclonal, Bioversion), thioredoxin (mouse monoclonal, Abcam), Vitamin D3 upregulated protein-1 (VDUP-1, rabbit polyclonal, Zymed Laboratories), phospho-ERK1/2 (rabbit polyclonal, Cell Signaling), phospho-p38MAPK, (rabbit polyclonal, Cell Signaling), proliferating cell nuclear antigen (PCNA) (mouse monoclonal, Sant Cruz), cyclin-dependent kinase 4 (CDK4) (mouse monoclonal, Santa Cruz) and p21cip1 (rabbit polyclonal, Santa Cruz). Immunoreactive bands were visualized using HRP-conjugated anti-rabbit or anti-mouse IgG (Sigma) and ECL western blot analysis system (Pierce). The membranes were then stripped and reprobed with β-Actin (mouse monoclonal, Sigma) to verify equal loading. The optical density of bands was quantified by ImageQuant Software (Molecular Dynamics, Sunnyvale, California, USA) and expressed as percentage of control.

**Thioredoxin Activity Assay**
The modified insulin disulfide reduction assay was performed to measure thioredoxin activity as described (30,31). In brief, 50 μg of lysed cells in lysis buffer (20mM Hepes, pH7.9, 100mM KCl, 300mM NaCl, 10mM EDTA, 0.1% nonidet P-40, 2mM Na3VO4, 1mM PMSF, 1μg/ml leupeptin, 1μg/ml aprotinin, and 1μg/ml pepstatin) were preincubated with 2μl of DTT activation buffer (50mM Hepes, pH7.6, 1mM EDTA, 1mg/ml BSA, 2mM DTT) at 37°C for 20 minutes in a total volume of 50μl to reduce thioredoxin. Then 30μl of reaction mixture containing 200μl of 1M Hepes, pH7.6, 40μl of 0.2 M EDTA, 40μl of 40mg/ml NADPH, and 500μl of 10mg/ml insulin were added. The reaction was started by addition of 5μl of rat thioredoxin reductase (Sigma) and incubated at 37°C for 20 minutes. An equal volume of water was added to the control samples. The reaction was stopped by the addition of 0.5ml of 6M guanidine-HCl and 1mM 3-carboxy-4-nitrophenyl disulfide (DTNB). The absorbance was measured at 412 nm and results were expressed as percentage of control.

**Statistical Analysis**
Experiments were repeated 3-6 times in duplicate or triplicate. Data are expressed as mean±SEM and analyzed by ANOVA or by unpaired Student’s t-test as appropriate. p<0.05 was considered significant.

**Figure legends**

**Figure S1.** Expression of NAD(P)H oxidase subunits, gp91phox (Fig S1A) and the small G protein Rac-1 (Fig S1B) in monoculture (MC) and coculture (CC) VSMC. VSMCs were cocultured with ECs for 72 hours and were analyzed by immunoblotting with antibodies recognizing human gp91phox and Rac-1. Data are expressed as percentage of control (MC) with control taken as 100%. Results are mean±SEM of 6 independent experiments.

**Figure S2.** Expression of phospho-specific ERK1/2 and p38MAPK in monoculture (MC) and coculture (CC) VSMC. Data are presented as pERK1/2 and p38MAPK:β-actin. Results are mean±SEM of 6 independent experiments.
Figure S1

A. gp91phox

β-actin

Rac1

β-actin

B. Rac1

gp91phox expression (% of control)

MC CC

Rac1 expression (% of control)

MC CC
Figure S2

**Western Blot Analysis**

- **p-ERK1/2** (Thr202/Tyr204)
  - 44KD
  - 42KD
  - β-actin
  - 42KD

- **pp38MAPK** (Thr180/Tyr182)
  - 38KD
  - 42KD
  - β-actin
  - 42KD

**Bar Graphs**

- **p-ERK1/2** (% of control): MC vs. CC
- **pp38MAPK** (% of control): MC vs. CC