Rac-Dependent Monocyte Chemoattractant Protein-1 Production Is Induced by Nutrient Deprivation

Neuza H.M. Lopes, Sanjay S. Vasudevan, David Gregg, Balakrishnan Selvakumar, Patrick J. Pagano, Herve Kovacic, Pascal J. Goldschmidt-Clermont

Abstract—Under ischemic conditions, the vessel wall recruits inflammatory cells. Human aortic endothelial cells (HAECs) exposed to hypoxia followed by reoxygenation produce monocyte chemoattractant protein-1 (MCP-1); however, most experiments have been performed in the presence of nutrient deprivation (ND). We hypothesized that ND rather than hypoxia mediates endothelial MCP-1 production during ischemia, and that the small GTP-binding protein Rac1 and reactive oxygen species (ROS) are involved in this process. ND was generated by shifting HAECs from 10% to 1% FBS. Superoxide production by HAECs was increased 6 to 24 hours after ND, peaking at 18 hours. MCP-1 production was increased over a similar time frame, but peaked later at 24 hours. These effects were blocked by treatment with antioxidants such as superoxide dismutase mimetic and N-acetylcysteine (NAC), or NADPH oxidase inhibitors, DPI and gp91ds-tat. Superoxide and MCP-1 production were enhanced by RacV12 (constitutively active) in the absence of ND, and were inhibited by RacN17 (dominant-negative) adenoviral transduction under ND, suggesting that the small G-protein Rac1 is required. In conclusion, ND, an important component of ischemia, is sufficient to induce MCP-1 production by HAECs, and such production requires a functional Rac1, redox-dependent pathway. (Circ Res. 2002;91:798-805.)

Key Words: monocyte chemoattractant protein-1 ■ superoxide ■ Rac ■ nutrient deprivation ■ ischemia

Severe ischemia is a common cause of tissue injury after organ transplantation and myocardial infarction and also promotes the development of ventricular dysfunction and atherosclerosis.1–3 Although most of the consequences of ischemia have been attributed to hypoxia or to postischemic reoxygenation,4–8 loss of blood flow is also the cause of nutrient deprivation (ND), which by itself may trigger multiple intracellular signaling pathways, independent of hypoxia or reoxygenation. After an episode of ischemia, inflammatory cells accumulate within blood vessels and play an important role in the progression of ischemic heart disease.7 Recent studies have suggested that reactive oxygen species (ROS) may be an important intracellular signaling messenger linking tissue ischemia to the subsequent inflammatory responses.8,9

Monocyte chemoattractant protein-1 (MCP-1), a chemotactic and activating chemokine peptide, is a key regulator of monocyte10 and T lymphocyte recruitment to sites of inflammation.11 In addition to its chemotactic properties, MCP-1 also induces functional changes in monocytes, including superoxide production, cytokine secretion, and adhesion molecule expression.11 Elevated levels of MCP-1 have been found in patients with unstable angina,12 myocardial infarction,13 and heart failure,14 as well as after coronary angioplasty,15 and have been correlated with poor outcomes in these conditions. However, the exact molecular mechanism controlling expression of MCP-1 in ischemia remains unclear.

The small GTP-binding protein Rac1 regulates the production of ROS by the NADPH oxidase complex, the major regulated source of superoxide in vascular cells.16–20 Previously, we have shown that Rac1 mediates oxidant production in response to hypoxia/reperfusion in several cells types17 as well as the induction of an acute inflammatory response in a mouse model of hepatic ischemia/reperfusion.21 The role of Rac1 and ROS in ischemia-induced MCP-1 production, however, has not been reported.

In this study, we now investigate the effects of ND, a condition associated with ischemia, on MCP-1 protein production by human aortic endothelial cells (HAECs) and the role of Rac1 in this process. We show that ND, but not hypoxia, induces MCP-1 production by HAECs in a time-dependent manner, which peaks at 24 hours. MCP-1 production under ND requires Rac1 and superoxide, demonstrated by inhibition with a dominant-negative Rac1 or with antioxidant treatment. In addition, ND stimulates nuclear factor-κB (NF-κB) transcriptional activity, leading to subsequent MCP-1 production through a Rac1-controlled pathway.
Materials and Methods

Cell Culture
HAECs were obtained from Clonetics. The cells (passage 6 to 8) were grown in endothelial growth medium (EGM-2-MV, Clonetics) supplemented with 10% of fetal bovine serum (FBS), gentamicin (50 μg/ml), and growth supplements until confluent monolayers were obtained. Then they were subjected to ND, by shifting from 10% to 1% of FBS and growth supplements for different time points indicated in each experiment. For the hypoxia/reoxygenation experiments, HAECs were placed in a modular hypoxia chamber (Billups-Rothenberg). All cultures were maintained in 95% air, 5% CO2 at 37°C.

Adenoviral Construction and Infection
The recombinant replication incompetent adenovirus RacN17 and RacV12, containing the c-Myc-tagged dominant-negative and the c-Myc-tagged constitutively activated form of Rac1, were prepared as described.22

Western Blotting Analysis
Protein lysates were prepared and extracts were separated by SDS-PAGE on a 4% to 20% Tris-glycine gel. Blots were then probed with monoclonal antibody to c-Myc.

Quantification of MCP-1 Protein
MCP-1 protein released into the medium was measured with a Quantikine Human MCP-1 Immunoassay ELISA kit (R&D Systems).

Reactive Oxygen Species Detection
ROS generation was estimated by dihydroethidium (DHE) staining using a Nikon Eclipse 800 fluorescence microscope, as described previously.23

Transfection and NF-κB Luciferase Reporter Assay
Transient transfection of the pNFκB-TATA reporter gene and pSV-B-gal was performed using a FUGENE 6 (Roche). Cellular activities of luciferase and β-galactosidase were performed according to the manufacturer’s protocol using a Luciferase Assay Kit (Promega) and Galacto-Light Chemiluminescent Reporter Gene Assay System (Tropix Inc).

Immunofluorescence
To study NF-κB nuclear translocation, HAECs under 6 hours of ND were stained with anti–NF-κB p65 polyclonal antibody (Santa Cruz) and anti-Myc Tag polyclonal antibody.

Rac1 Activation Assay
Rac1 activation was measured by affinity precipitation assay (Upstate), using a GST-PAK-PBD fusion protein that binds GTP-bound activated Rac1, as described.24

Statistical Analysis
Data are presented as mean±SEM. The results were compared by either ANOVA followed by Bonferroni’s multiple comparison post test or Student’s t test wherever appropriate. Statistical analysis was performed using STAT view software SAS Institute Inc and Altura Software Inc. Significance was defined as a value of P<0.05.

An expanded Materials and Methods section can be found in the online data supplement at http://www.circresaha.org.

Results

Nutrient Deprivation Induces MCP-1 Production by HAECs
Because MCP-1 has been shown to participate in inflammatory responses in ischemia/reperfusion models,7,25 we examined the effect of ND, a consequence of ischemia, to induce MCP-1 production on HAECs by ELISA. ND resulted in a significant increase in MCP-1 release by HAECs (5.8±0.7-fold) in a time-dependent manner peaking at 24 hours, in contrast to unstarved cells at a similar time point. ND was obtained by shifting the serum from 10% to 1%, measurements were quantified by ELISA, and values are represented as mean±SEM for 4 independent experiments (∗P<0.05, **P<0.01 vs control). B, HAECs were subjected to ND for 24 hours with different serum concentrations (0% to 2%). Maximum protein expression was noted at 0.1% of serum, relative to control cells in 10% serum. Values are represented as mean±SEM for 3 independent experiments (P<0.05, **P<0.01 vs control). C, HAECs subjected to hypoxia (Billups Chamber) demonstrated decreased MCP-1 with isolated hypoxia, but increased MCP-1 with ND or hypoxia followed by prolonged reperfusion (∗P<0.05 vs normoxia). Values are represented as mean±SEM for 3 independent experiments.

Figure 1. ND increases MCP-1 protein expression. A, MCP-1 protein expression increased in a time-dependent manner with maximum production at 24 hours, in contrast to unstarved cells at a similar time point. ND was obtained by shifting the serum from 10% to 1%, measurements were quantified by ELISA, and values are represented as mean±SEM for 4 independent experiments (∗P<0.05, **P<0.01 vs control). B, HAECs were subjected to ND for 24 hours with different serum concentrations (0% to 2%). Maximum protein expression was noted at 0.1% of serum, relative to control cells in 10% serum. Values are represented as mean±SEM for 3 independent experiments (P<0.05, **P<0.01 vs control). C, HAECs subjected to hypoxia (Billups Chamber) demonstrated decreased MCP-1 with isolated hypoxia, but increased MCP-1 with ND or hypoxia followed by prolonged reperfusion (∗P<0.05 vs normoxia). Values are represented as mean±SEM for 3 independent experiments.
Recent evidence has suggested that ROS play a pivotal role in mediating endothelial cell response to ischemia. For example, increased intracellular ROS levels can regulate redox-sensitive genes, such as MCP-1. Thus, to investigate whether ND-induced MCP-1 protein production is mediated via ROS generation, we measured the levels of the intracellular -O₂⁻ with the oxidant-sensitive probe, DHE. The levels of nuclear DHE fluorescence significantly increased after exposure to ND at 6 hours and reached a plateau at 18 hours (≈6-fold increase) (Figure 2). To elucidate the sources of ROS generation during ND, we used DPI (10 μmol/L), an inhibitor of flavin-containing oxidases and a more specific competitive inhibitor of NAD(P)H oxidase, gp91ds-tat (25 μmol/L). -O₂⁻ generation was markedly attenuated when the cells were pretreated with DPI or gp91ds-tat by 59.7% and 55.3%, respectively (Figure 2). Similarly, the SOD mimetic, MnTNPyP caused a significant reduction on DHE signal after ND. These effects suggest that ND-induced -O₂⁻ generation occurs through the activation of NADPH oxidase. In addition, to test a putative role for other sources in ROS generation in response to ND, nitric oxide synthase was inhibited by preincubation with L-NAME, 10 μmol/L and xanthine oxidase with allopurinol (100 μmol/L). In contrast to the NADPH oxidase inhibitors, L-NAME and allopurinol only modestly attenuated DHE oxidation after ND (Figure 1). This observation suggests that the involvement of NOS and the xanthine oxidase in DHE oxidation were relatively minor in our model.

**Effects of Antioxidants on ND-Induced MCP-1 Production**

To determine whether MCP-1 production under ND is mediated through superoxide, we examined the effects of various antioxidants/ROS scavengers on this response. Cells were incubated for 1 hour before ND with two different SOD mimetics, MnTNPyP and TEMPOL. In the presence of -O₂⁻ scavengers, there was a significant reduction on MCP-1 protein levels by 58.3% and 62.8% (Figure 3). A similar effect was observed when the cells were pretreated with the cell permeant chemical antioxidant, N-acetylcysteine (NAC). Next, we demonstrated the effect of NADPH oxidase inhibition on MCP-1 production under ND by preincubation with DPI and gp91ds-tat. Consistent with our results with antioxi-
dants, both inhibitors of the NADPH oxidase system effectively inhibited MCP-1 secretion by 60.4% and 53.9% (Figure 3). In contrast, L-NAME and allopurinol did not show relevant protective effect on MCP-1 production. These data indicate that superoxide/NADPH oxidase system mediates the enhanced release of MCP-1 during ND.

Role of Rac1-Mediated ROS Generation and MCP-1 Production

It is known that Rac1 is an integral part of the NADPH oxidase complex and regulates ROS production in phagocytic and nonphagocytic cells. In an effort to explore whether Rac1 is required for ROS generation and MCP-1 production after ND, we transduced HAECS with dominant-negative Rac1. Western blot analysis detected the expression of the epitope-tagged form of Rac1 in transduced cells (Figure 4). Adenoviral transduction led to gene transfer in 70% of infected cells, consistent with previous results in HAECS (data not shown). RacN17-transduced cells demonstrated 70.8% attenuation in DHE staining relative to AdNull-transduced cells after ND. Similar results were also observed with GGTI-298, a geranylgeranyl transferase inhibitor that blocks the prenylation of Rac, an important step in membrane translocation of Rac and subsequent NADPH oxidase activation (Figure 4). This suggests that Rac1-dependent pathways are responsible for the ND-induced \( \bullet O_2^- \) generation. Interestingly, transduction of HAECS with RacV12 even in the
NF-H9260 PAK fusion protein that binds to GTP-bound activated Rac. Pull down of the proteins bound to PAK agarose were separated by SDS-PAGE, transferred to nitrocellulose, and then probed with anti-Rac. As a positive control, cell lysates were loaded with GTPγS for 30 minutes before incubation with Pak beads. Western blot demonstrates that ND cells strongly activated Rac at 15 to 30 minutes and remained high up to 18 hours, compared with unstarved cells. Bottom, Blot shows equal amount of proteins in all above conditions before treatment with PAK beads. Results are representative of 3 independent experiments.

Using a PAK-affinity precipitation assay for Rac activation, we showed that ND strongly activates Rac after 15 to 30 minutes, and then induces a prolonged but low level of Rac1 activation, up to 18 hours (Figure 6). Taken together, these data indicate that Rac is required to trigger activation of vascular NADPH oxidase and subsequent MCP-1 protein expression in the context of ND.

**Role of NF-κB in the Regulation of MCP-1 Expression**

Because Rac1 is known to regulate the transcriptional activity of NF-κB and NF-κB activation is redox-sensitive, we speculated that Rac1-regulated intracellular ROS produced by ND may promote MCP-1 release by HAECS through NF-κB. Activation of NF-κB occurs with the phosphorylation and release of IκB protein, which forms an inhibitory complex with the NF-κB dimer in unstimulated cells, retaining NF-κB in the cytoplasm. On activation, NF-κB translocates to the nucleus, binds target DNA sites, and activates gene transcription. Thus, we followed the intracellular movement of NF-κB by immunofluorescence using NF-κB p65 specific antiserum in the presence of various redox cellular conditions after 6 hours of ND. We found marked nuclear translocation of NF-κB p65 in HAECS in response to ND (6 hours) as well as in cells transduced with RacV12. In contrast, control cells mostly retained NF-κB in the cytoplasm. In addition, absence of ND also resulted in an intense increase in DHE fluorescence in a gene dose-dependent fashion (Figure 4). We next assessed the inhibitory effect of RacN17 expression and GGTI-298 on MCP-1 protein production on ND. We observed a significant reduction by 54.3% and 67.9% in MCP-1 production after ND, with RacN17 and GGTI-298, respectively. In contrast, cells pretreated with FFI-277, a farnesyl transferase inhibitor specific for Ras activation, did not show significant reduction of MCP-1 production (Figure 5). Moreover, overexpression of RacV12 in unstarved cells increase MCP-1 expression to a similar level as that obtained with ND (Figure 5).

**Figure 6.** ND induces Rac activation. Top, Rac activation was demonstrated by a PAK-affinity precipitation assay using a GST-PAK-PBD fusion protein that binds to GTP-bound activated Rac. Pull down of the proteins bound to PAK agarose were separated by SDS-PAGE, transferred to nitrocellulose, and then probed with anti-Rac. As a positive control, cell lysates were loaded with GTPγS for 30 minutes before incubation with Pak beads. Western blot demonstrates that ND cells strongly activated Rac at 15 to 30 minutes and remained high up to 18 hours, compared with unstarved cells. Bottom, Blot shows equal amount of proteins in all above conditions before treatment with PAK beads. Results are representative of 3 independent experiments.

**Figure 7.** ND induces NF-κB nuclear translocation. A, Immunofluorescence staining of NF-κB p65 in ND-treated HAECS shows increased nuclear translocation of NF-κB p65, relative to unstarved cells. RacN17-transduced cells at 25 MOI under similar conditions demonstrated a significant reduction in NF-κB p65 nuclear translocation, in contrast to ND cells. RacV12 (100 MOI)-transduced cells under unstarved conditions showed an increased nuclear translocation of NF-κB p65, similar to ND cells. b and d, Absence of nuclear NF-κB p65 is observed in unstarved cells (b) and RacN17 transduced cells under ND (d). c and f, Nuclear localization of NF-κB p65 is observed in ND cells (c) and RacV12 transduced cells (f). e and g, Expression of Rac in HAECS using an anti-myc monoclonal antibody in RacN17 (e) and RacV12 (g) transduced cells. Results are representative of 2 independent experiments. Percent of NF-κB p65 nuclear translocation was determined by counting 100 to 200 cells per condition.

RacN17-transduced cells showed a 70.3% reduction in NF-κB nuclear translocation under ND (Figure 7).

Next, to determine whether ND also increased NF-κB-dependent transcriptional activity, NF-κB reporter assays were performed. ND induced NF-κB transcriptional activity in a time-dependent manner, peaking at 18 hours, an ~4-fold increase relative to control (Figure 8). Moreover, RacN17, as well as pretreatment with SOD mimetic or the antioxidant NAC, substantially reduced NF-κB transcription activity. On the other hand, transduction with RacV12 significantly increased NF-κB activity, even in the absence of ND. Therefore, ND-induced NF-κB nuclear translocation and transcriptional activity were consistent and appeared to be dependent on Rac1-regulated ROS production.
To further confirm that NF-κB activity is indeed involved in the regulation of MCP-1 protein production by ND, we pretreated the cells with inhibitors of the NF-κB pathway, including PDTC, which inhibits IκB degradation, and the more selective inhibitor, SN-50, which contains the nuclear localization signal of the p50 subunit and interferes with the nuclear transport of NF-κB.33 Both agents significantly reduced MCP-1 protein production by ND (Figure 8). These results confirm that NF-κB under the control of Rac1 and NADPH oxidase contributes to expression of MCP-1 protein under ND conditions.

Discussion

This study provides new evidence that nutrient, and perhaps, growth factor deprivation, conditions associated with ischemia, induce MCP-1 production by HAECs in a time-dependent fashion. This process is redox-sensitive, involves NADPH oxidase activation and subsequent ROS generation, and is regulated by the small GTPase Rac1. Moreover, we show that NF-κB is a Rac-dependent downstream regulator of MCP-1 expression. MCP-1 released by endothelial cells in a canine model of ischemia has been shown to contribute to recruitment of monocytes7,25 and blocking antibodies against MCP-1 can reduce infarct size in rat hearts.34 In our model, the increase in MCP-1 protein concentration in response to ND is a robust 5-fold induction, far exceeding the 2-fold increase seen in clinical studies of myocardial infarction,13 unstable angina,12 or restenosis.15 Furthermore, the overexpression of MCP-1 resulting from ND reaches levels that optimally stimulate chemotaxis.35,36 In contrast, hypoxia if anything, decreases MCP-1 production by HAECs, strongly suggesting that ND, and not hypoxia, is the crucial component of ischemia that triggers signaling pathways that lead to inflammatory responses under ischemic conditions. Consistent with our findings, previous studies in pulmonary endothelial cells using an in vitro oxygenated ischemia model, have demonstrated production of ROS and activation of signaling pathways in response to combined shear stress and ND.27,36,37 In addition, studies have also shown decreased production of ROS by hypoxia in pulmonary endothelial cells.38,39 Thus, for the first time, we demonstrated that ND is sufficient to induce ROS generation and MCP-1 production by HAECs under normoxia condition.

We have shown that ND-induced ROS production is via the NADPH oxidase complex. Although ischemia and reperfusion are known to increase endothelial cell ROS production, the sources of ROS in global ischemia are unclear and pretreated with NAC (μmol/L) and MnTNPyP (μmol/L) inhibited NF-κB-dependent gene transcription, relative to nonpretreated cells. RacV12 cotransduced cells under un-starved conditions demonstrated a significant increase in NF-κB transcriptional activity similar to cells in ND conditions. Values represent the mean±SEM for 3 independent experiments (*P<0.05 vs ND cells alone, **P<0.01 vs unstarved cells). C, HAECs were pretreated with NF-κB inhibitors, NF-κB SN50, and PDTC (μmol/L) for 60 minutes before ND. NF-κB inhibitors demonstrated a significant reduction in MCP-1 protein levels, relative to ND cells pretreated with scramble peptide (NF-κB SN50M). Values represent the mean±SEM for 3 independent experiments (*P<0.05 vs ND cells alone).

Figure 8. ND induces NF-κB-dependent gene transcription. A, HAECs were transfected with NF-κB reporter construct plus pSVβ-gal to control for transfection efficiency. Thirty-six hours after transfection, cells were subjected to ND. ND induced NF-κB transcriptional activity in a time-dependent manner with maximum activity at 18 hours, estimated by luciferase activity. Results are expressed as increase in luciferase activity, relative to unstarved cells. Values represent the mean±SEM for 3 independent experiments (*P<0.05, **P<0.01). B, RacN17 (MOI)-cotransduced HAECs under ND conditions demonstrated a significant reduction in NF-κB transcriptional activity, in contrast to AdNull (MOI)-transduced cells under similar conditions. ND cells
may depend on the different components of ischemia. Previously, we and others have shown NADPH oxidase is the source of ROS in vascular cells after hypoxia/reperfusion injury.17,39 In contrast, some studies have shown that xanthine oxidase or endothelial nitric oxide synthase are key source of ROS in anoxia/reoxygenation models.37,40,41 We now show in HAECs that after 6 hours, ND significantly increases NADPH oxidase-dependent superoxide production, as assessed by DHE fluorescence. The ability of DPI and the gp91 ds-tat peptide, but not L-NAME or allopurinol, to decrease DHE fluorescence and MCP-1 production in response to ND strongly suggest that NADPH oxidase is the major source of superoxide in our model. Similarly, Fisher and colleagues,28 using a lung-oxygenated ischemic model, have shown inhibition of ROS generation with DPI, but not with allopurinol, a xanthine oxidase inhibitor. Furthermore, in vivo ROS production after ischemia is decreased in the gp91<sup>phox</sup> knockout mouse, confirming the role of NADPH oxidase role in oxygenated ischemia.28

Ischemia is a complex physiological process, in which both hypoxia and deprivation of nutrients and growth factors contribute to cellular injury. Although it has been shown that ischemia/reperfusion results in inflammatory responses, such as an MCP-1 increase, the model systems used in most of these studies have included both hypoxia and ND to recapitulate the ischemic insult. We have dissected the role of ND and hypoxia during ischemic events relative to MCP-1 production. Our results indicate that ND is sufficient to trigger MCP-1 overexpression during ischemic events, independent of hypoxia or reoxygenation. However, we cannot rule out the possibility of independent effects of hypoxia in this process. Other aspects of ischemia, such as shear stress or lack thereof, were not examined. The ability of ND, however, to activate the NADPH oxidase and NF-κB/MCP-1 pathway in vitro suggests that this pathway may mediate ischemia-induced inflammatory response in vivo as well.

Further, NADPH oxidase activity requires Rac, and we demonstrate for the first time a requirement of Rac1 in an intracellular pathway leading to ROS generation after ND. We showed strong Rac activation using a PAK-binding assay, after 15 to 30 minutes of ND, with subsequent lower amplitude but prolonged Rac activation. Interestingly, Rac activation as measured by PAK binding precedes the detection of Rac-dependent superoxide production. This illustrates the ability of Rac to be differentially activated for unique effector pathways and highlights the limitation of using PAK binding as a surrogate for Rac activity because it only measures Rac affinity for one effector, PAK. The requirement of Rac in ND-induced MCP-1 production, however, is clear. Rac inhibition with a dominant-negative construct or GGTI leads to a decrease in ND-induced NF-κB activation and MCP-1 production by HAECs. Moreover, cells overexpressing the constitutively active form of Rac1 mimic the effect of ND with increased superoxide, NF-κB activation, and subsequent MCP-1 production. The kinetics of NF-κB transcriptional activity occurred concurrently with the Rac-induced superoxide generation and later than Rac/PAK-binding, with NF-κB translocation at 6 hours and reporter activity beginning at 6 hours and peaking at 18 hours. Together, these data support that Rac is required to regulate activation of vascular NADPH oxidase, NF-κB activation, and subsequent MCP-1 protein expression in the context of ND.

ROS other than superoxide or additional signaling pathways such as MAP kinase may also participate in ND-induced MCP-1 production. Nonetheless, our data suggest that efforts aimed at preventing inflammatory injury during ischemic events, based on targeted inhibition of Rac or Rac-dependent pathways, may be an effective and useful approach in a variety of clinical conditions in which the ischemia and MCP-1 play a crucial role.

In conclusion, the data presented here provide evidence for the requirement of Rac1/NADPH oxidase signaling pathway in mediating superoxide generation, NF-κB activation, and subsequent MCP-1 production in response to reduced nutrient supply, one of the components of ischemia, independent of hypoxia or reoxygenation.

Acknowledgments
This study was supported by NIH grant ROI HL-71536 to Dr Goldschmidt-Clermont. We thank Dr Christopher D. Kontos for his helpful comments.

References


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Circ Res. 2002;91:798-805; originally published online October 3, 2002;
doi: 10.1161/01.RES.0000040421.54108.81

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/91/9/798

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Material and Methods

Reagents:

Diphenylene iodonium chloride (DPI), allopurinol, and N-nitro-l-arginine methyl ester (L-NAME) were obtained from Sigma. TEMPOL, MnTNPyP, Geranylgeranyltransferase inhibitor (GGTI-298), farnesyltransferase inhibitor (FFT1-277), NFkB SN50 cell inhibitor peptide, and NFkB SN50M cell-inactive control peptide were purchased from Calbiochem. Dihydroethidium (DHE) was from Molecular Probes. N-acetylcysteine (NAC) was purchased from American Reagent Laboratories. GP91 ds-tat and Scramb-tat peptides were synthesized by Dr. P.J. Pagano, as described \(^1\). All other chemicals and reagents were obtained from Gibco.

Cell Culture:

Human aortic endothelial cells (HAEC) were obtained from Clonetics. The cells (passage 6-8) were grown in endothelial growth medium (EGM2-MV, Clonetics) supplemented with 10% of fetal bovine serum (FBS), gentamicin (50 ug/ml) and growth supplements until confluent monolayers were obtained. They were then subjected to ND, by shifting from 10% to 1% of FBS and growth supplements for different time points indicated in each experiment. For the hypoxia/reoxygenation experiments HAEC were placed in a modular hypoxia chamber (Billups-Rothenberg). The chamber was subsequently flushed for 30 minutes with 95% N\(_2\)/5% CO\(_2\) gas mixture, which is known to produce air O\(_2\) tension of less than 10 Torr. \(^2\). The cells were then returned to a 37°C
incubator for 18 hours. At the end of hypoxic period, the chamber was removed from the incubator and opened to room air and the supernatant and the cells were collected. In some experiments the hypoxia media was rapidly replaced with oxygen-saturated medium and the MCP-1 levels were assessed at 6, 12 and 24 hours after reperfusion. All cultures were maintained in 95% air, 5% CO₂ at 37°C.

**Adenoviral Construction and Infection:**

The recombinant replication incompetent adenovirus RacN17 and RacV12, containing the c-Myc-tagged dominant negative and the c-Myc-tagged constitutively activated form of Rac1, were prepared as described (3). HAEC in 2% FBS were seeded 24 hours before infection at a density of 200 cells/mm². Adenoviral transduction was performed at the desired multiplicity of infection (MOI) overnight, after which the medium was replaced by fresh EGM-2MV with 10% of FBS. The cells were incubating for 36 hours and were treated as indicated. The control adenovirus dl312 (AdNull) lacking the cDNA insert, was used as a control for adenoviral transduction.

**Western Blotting Analysis:**

Protein lysates were prepared and extracts were separated by SDS-PAGE on a 4-20% Tris-glycine gel. After transferring to nitrocellulose, blots were probed with mouse monoclonal antibody to c-Myc (9E10, Upstate Biotechnology), followed by incubation with corresponding horseradish peroxidase-conjugated secondary antibody (Jackson Laboratories). Immunoreactivity was visualized with enhanced luminescence. (Pierce Chemical Co.)
Quantification of MCP-1 Protein:

MCP-1 protein released into the medium was measured with a Quantikine Human MCP-1 Immunoassay ELISA kit (R&D Systems). Culture supernatants were collected at different time points after ND, and ND-induced MCP-1 production was assessed. Each sample was measured in duplicate and MCP-1 levels are expressed as an average of these values normalized to protein concentrations. Protein concentration was estimated using bicinchoninic acid protein assay (Pierce Chemical Co).

Reactive Oxygen Species Detection:

ROS generation was estimated by DHE staining using a Nikon Eclipse 800 fluorescence microscope, as describe previously (4). When oxidized to ethidium upon reaction with superoxide (‘O₂’), ethidium binds to DNA, resulting in increase in quantum yield. After ND, the cells were loaded with DHE at concentration of 10 μmol/L in Hanks Balanced Salt Solution (HBSS) for 30 minutes at 37°C in 5% CO₂. At the end of incubation, the monolayer was rinsed with HBSS, and the cells were examined alive at 488 nm excitation and 610 nm emission wavelength. Digital images were recorded on a SenSys digital camera. The fluorescence intensity was quantified on a gray scale of 0-255 and measured using MetaMorph software. The DHE fluorescence intensity was calculated by dividing the total integrated optical density by the total number of cells in each field and is expressed as arbitrary units.
Transfection and NFκB Luciferase Reporter Assay:

Transient transfection of the NFκB reporter gene was performed using a FUGENE 6 (ROCHE). HAEC were seeded at density of 300 cells/mm² and grown in complete media for 24 hours before transfection with 2.2 μg of DNA including 2 μg of pNFκB-TA luciferase promoter reporter and 0.2 μg of pSV-B-gal (Promega) in complete media for 6 hours. Following removal of the transfection mixture, cells were maintained in EGM-2MV with 10% FBS for 36 hours prior to indicated treatments. Cellular activities of luciferase and β-galacosidase were performed according to the manufacturer’s protocol using a Luciferase Assay Kit (Promega) and Galacto-Light Cheminuminescent Reporter Gene Assay System (Tropix,Inc.) using a Berthold Lumat LB9501 luminometer. Activity of the NF-κB promoter-reporter construct was normalized to the activity of the β-gal reporter. When cotransduced with adenovirus, the reporter transfection was performed 18 hours after the transduction with the indicated adenovirus.

Immunofluorescence:

To study NFκB nuclear translocation, HAEC were stained with anti-NFκB p65 polyclonal antibody (Santa Cruz) and anti-Myc Tag polyclonal antibody. Cells on cover slips were fixed and permeabilized with acetone:alcohol for 10 min at −20°C. After blocking with 5% (g/ml) milk for 30 min, cells were incubated overnight at 4°C with anti-p65/rel polyclonal and anti Myc-Tag polyclonal antibodies (1:100). Texas red-conjugated goat-anti-rabbit IgG or FITC-cojugated goat-anti-mouse IgG were used as secondary antibodies at 1μg/ml for 2 hours at room temperature. Cell nuclei were
counterstained with Hoechst 3342 (5μg/ml) for 10 min at 4° C. Immunoreactivity was visualized with a Nikon E800 fluorescent microscope.

**Rac1 Activation Assay:**

Rac1 activation was measured by affinity precipitation assay (Upstate), using a GST-PAK-PBD fusion protein that binds GTP-bound activated Rac1, as described \(^5\). Cells were lysed with magnesium lysis buffer and the supernatant was incubated on ice with the PAK-PBD fusion protein bound to glutathione agarose beads for 1 hour. Protein complexed to the beads were recovered by centrifugation, washed twice with the lysis buffer, and resuspended in 2x laemmli sample buffer. As a positive control, cell lysates were loaded with GTP\(_{\gamma}\)S for 30 min prior to incubation with PAK-PBD beads. The protein were resolved by 4-20% SDS-PAGE and transferred to membrane. The membranes were then probed with anti-Rac1 antibody (Upstate, 1:1000).

**Cell viability assay:**

Cell death was measured by percentage of trypan blue positive cells. HAEC with 10% of serum and under various conditions of ND were studied for viability after 24 hours. We also assessed the viability of HAEC following treatment with different antioxidants and adenoviruses exposure under ND where indicated. Results represent the average of at least two experiments each, done in triplicate.
Statistical Analysis:

Data are presented as mean ± S.E.M. The results were compared by either ANOVA following by Bonferroni’s multiple comparation post-test or Student t test wherever appropriate. Statistical analysis was performed using STAT view software SAS Institute Inc. and Altura Software Inc. Significance was defined as a value of p < 0.05.

References:


Figure and Legend:

Figure 1: Percentage of cell death in HAEC under ND. (a) Cell death, as measured by percentage of trypan blue positive cells, in HAEC under various conditions of ND. Increasing cell death was observed in a FBS concentration-dependent manner. (b) HAEC transduced either with the constitutively active mutant of Rac, RacV12, with the dominant negative, RacN17, or Adnull control were submitted to ND for 24 hours and cell death analysis were performed. Both Rac mutant forms demonstrated a similar degree of apoptosis after ND with about 6%. p<0.05. (c) Where indicated, cells were incubated with TEMPO (M) MnTyrPT (μM/L) NAC (μM/L) GGTI-298 (μM/L) DPI (μM/L) and gp91 ds-tat (μM/L) throughout 24 hours of ND. The levels of cell death reduced from 9.9% to 4; 4.6; 6; 5.8 and 5.5% respectively. p<0.05.
Online Figure 1

(a) % cell death vs. FBS %

(b) % cell death vs. ND(24 hours) with different Adenovirus conditions:
- AdNull
- AdNull
- Rac V12
- Rac N17
Figure 1

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<th>MnTNPyp</th>
<th>TEMPOl</th>
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