Interleukin-1 and Nitric Oxide Increase NADPH Oxidase Activity in Human Coronary Artery Smooth Muscle Cells

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Key Words

Nitric oxide · NADPH oxidase · Interleukin-1 β · Coronary artery smooth muscle cells · Smooth muscle superoxide

Abstract

Objective: Cytokines, nitric oxide (NO) and reactive oxygen species (ROS) are well known for their pathogenic effects in development of cardiovascular diseases. Interleukin-1 β (IL-1 β) is known to induce NO generation, however it is not well established if IL-1 β or NO regulate production of ROS, such as superoxide anion. Therefore, the main objective of this study was to evaluate the effect of IL-1 β or NO on enzyme activity of NADPH oxidase (NOX), a superoxide-generating system recently documented to participate in a variety of vascular functions. **Methods:** Human coronary artery smooth muscle cells (SMC) obtained from Clonetics were treated with IL-1 β and NO donor, sodium nitroprusside (SNP), in culture. Nitrites accumulated in supernatants of SMC cultures were measured as an index of NO released following treatment with IL-1 β . NOX enzyme activity was assayed using cytochrome c as the electron acceptor. **Results:** Treatment with IL-1 β resulted in a 3-fold increase in the production of NO by SMC. Both IL-1 β and SNP enhanced NOX activity, by 67 and 45%, respectively, following 24 h of treatment. **Conclusion:** This study suggests that NO or NO-

generating cytokines might regulate the production of ROS in the cardiovascular system through modulation of superoxide-generating systems such as NOX.

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Introduction

Cytokines, reactive oxygen species (ROS) and nitric oxide (NO) have emerged as important mediators of inflammatory processes during atherogenesis [1–3]. Among cytokines interleukin-1 β (IL-1 β) has been shown to be a potent inflammatory effector that induces NO overproduction through NF κ -B-mediated increase in inducible NO synthase (NOS) gene expression and contributes towards vascular pathology [4, 5]. NO is also now known to be involved in a number of pathophysiological conditions either through its property as a reactive nitrogen or oxygen species or through its interaction with guanylate cyclase [6, 7]. In vascular tissues, NO is produced through constitutive (eNOS) or inducible (iNOS) NO synthases and has been shown to be influenced by cytokines, growth factors and ROS, such as hydrogen peroxide and superoxide anion [8–10]. ROS are excessively produced along with pro-inflammatory cytokines such as interleukins and tumor necrosis factor, during atherosclerosis [5, 11, 12]. Superoxide anion production in vascular tissue is

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1011–7571/04/0131–0026\$21.00/0

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carried out by xanthine oxidases, cyclooxygenases and NADPH oxidases (NOX). Recent work has shown that NAD(P)H oxidases are the major sources of superoxide in vascular cells and are essential in both physiological responses such as cell growth, cell migration and modification of the extracellular matrix, and in pathological states associated with uncontrolled growth and inflammation, such as atherosclerosis [13]. NO generation has been well documented to be influenced by cytokines and ROS, however, it is not clearly established if three known species of NO, namely NO[•], NO⁻ and NO⁺ that share a common mechanism of signaling in biological systems through activation of guanylate cyclase, regulate the production of ROS [14]. Activation of eNOS and IL-1 β have been linked to superoxide anion and ROS production [15, 16], respectively, however, it remains to be established if NO (radical, cation or anion) released by NO donors such as sodium nitroprusside (SNP) or by IL-1 β modulates enzyme systems responsible for superoxide production. This study was, therefore, undertaken to evaluate the effect of IL-1 β and SNP (NO⁺ donor) on NADPH oxidase enzyme activity in human coronary artery smooth muscle cells (SMC).

Materials and Methods

Materials

Cytochrome C, bovine serum albumin, penicillin/streptomycin and fetal calf serum were purchased from Sigma Chemical Company (St. Louis, Mo., USA) whereas IL-1 β was procured from Calbiochem (La Jolla, Calif., USA). DMEM-Ham's F-12 (1:1) and trypsin-EDTA were from GIBCO (Grand Island, N.Y., USA), and a growth medium (SmGm2) was obtained from Clonetics (BioWhittaker Inc., Walkersville, Md., USA). Human coronary artery SMC were also obtained from Clonetics. All other reagents were of highest quality available and purchased from Sigma or Calbiochem.

Cell Culture

Human coronary artery SMC were obtained at passage 2 and were grown in growth medium (Clonetics) in a humidified atmosphere of 5% CO₂-95% air. For all experiments SMC (at passage 3) were seeded in 6-well plates at a density of 1.5×10^5 cells/well and were cultured for 3–5 days in growth medium (SmGm2) before the start of an experiment. SmGm2 medium was supplemented with 10% fetal calf serum and penicillin/streptomycin (5 U, 5 μ g/ml, respectively).

NOX Assay

Following 24-hour treatment of SMC with IL-1 β or SNP, NOX activity was measured at room temperature by a modification of the method of Pagano et al. [17] using cytochrome c as the electron acceptor. Briefly, cell cultures were growth-arrested (with serum free DMEM F-12 medium) for 24 h and then treated for 24 h with IL-1 β or SNP in the presence of DMEM F-12 supplemented with 0.1%

Fig. 1. Effect of IL-1 β on NOX activity in cultured SMC from human coronary artery. Values are mean $\pm$ SD of four different experiments and enzyme activities are shown as percent of control. (NOX activity in control SMC was 2.95 ± 0.11 nmol/min/mg protein.)

fetal bovine serum. Following treatment, NOX activity was assayed in cell homogenates by adding 25–50 μ g protein to the reaction mixture (100 μ M cytochrome c and 0.25 mM NADPH in 0.1 M sodium phosphate buffer, pH 7.0) in the presence or absence of 25 μ M diphenyliodonium, an inhibitor of NADPH oxidase. Diphenyliodonium-inhibitable activity was taken as the NADPH oxidase activity.

Measurement of Nitrites

Supernatants of SMC cultures were collected following treatment with IL-1 β and nitrite concentration was measured in each supernatant as an index of NO production. Briefly, 300 μ l of culture supernatant was allowed to react with 200 μ l of Greiss reagent [18] and incubated at room temperature for 15 min. Optical density of each sample was measured spectrophotometrically at 570 nm and nitrite concentrations were calculated using fresh culture medium as blank and NaNO₂ as a standard.

Results

To examine the effect of IL-1 β on NOX, SMC were treated with 25 U/ml and 50 U/ml of IL-1 β for 24 h. These two concentrations of IL-1 β have been used by other workers in similar studies [19]. Both concentrations of IL-1 β resulted in a marked increase (50–65%) in the NOX activity (fig. 1). IL-1 β (at 25 U/ml and at 50 U/ml concentrations) also markedly increased (2- to 3-fold) the nitrite concentration in supernatant of SMC cultures (fig. 2), suggesting that the potentiation effect of IL-1 β on NOX might be mediated through NO. To examine the effect of NO on NOX, SNP, a well-known donor of NO, was added to the SMC cultures at 50 and 100 μ M concentrations. SNP has been used as an NO donor in previous studies,

Fig. 2. Nitrite levels in supernatants from SMC cultures treated with IL-1 β . Values are mean $\pm$ SD of four different experiments.

Fig. 3. Effect of SNP, an NO donor on NOX activity in cultured SMC from human coronary artery. Values are mean $\pm$ SD of four different experiments and enzyme activities are shown as percent of control. (NOX activity in control SMC was 2.95 ± 0.11 nmol/min/mg protein.)

however, it has been shown to have toxic effects on various cell cultures when added at concentrations higher than 100μ M [20]. Both concentrations of SNP resulted in a significant increase (40%) in NOX activity (fig. 3). IL-1 β was observed to be more potent than SNP in enhancing the NOX activity.

Discussion

Growth and function of arterial smooth muscle cells are influenced greatly by cytokines, growth factors and ROS during atherogenesis [21–24]. IL-1 β has been shown

previously to induce production of NO, a reactive nitrogen (oxygen) species through activation of iNOS. Increased NO generation indicated by 2- to 3-fold accumulation of nitrite in supernatants of SMC cultures treated with IL-1 β supports earlier findings that IL-1 β induces NO generation [8]. IL-1 β -stimulated iNOS induction and resultant overproduction of NO has been reported to be potentiated by superoxide anion [10]. However, the influence of IL-1 β on generation of oxygen free radical species, such as superoxide anion, is poorly understood. A marked increase in NOX activity of SMC following treatment with IL-1 β in the present study suggests that activation of NADPH oxidase could be one of the mechanisms for overproduction of ROS in response to IL-1 β treatment. Tolando et al. [25] have shown previously that IL-1 β and tumor necrosis factor induce release of superoxide anion and hydrogen peroxide in endothelial cells, suggesting a possible role of NOX in ROS production. Our study clearly demonstrates that IL-1 β causes an increase in NOX activity in SMC and suggests a potential new mechanism for ROS-stimulated SMC proliferation during atherogenesis, because superoxide anions have been shown to promote SMC growth [26]. Though NO and ROS have been shown to interact in more than one way [27, 28], the effect of NO on ROS production has not been clearly established. Enhancement of NADPH oxidase activity by SNP, an NO donor, observed in this study reveals that NO might be regulating some cellular functions through modulation of NOX-mediated superoxide anion production. Induction of NOX activity by IL-1 β could also be NO-mediated since IL-1 β has been found to increase NO generation. However, our results show that induction of NOX activity by IL-1 β is stronger than that induced by an NO donor, SNP. Therefore IL-1 β -induced increase in NOX activity might involve other mechanisms besides NO-mediated processes. Our results strongly suggest that both IL-1 β and NO enhance NOX activity in coronary artery smooth muscle cells and this might play an important role in vascular biology and pathogenesis of cardiovascular diseases.

Conclusion

Our study shows that NO and NO-generating cytokines enhance NOX activity in cultured coronary artery SMC. IL-1 β and NO might regulate a number of cardiovascular functions through modulation of superoxide-generating systems such as NOX, and contribute to atherogenesis by regulating ROS production.

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