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Nitric Oxide Donor, NOR-3, Increased Expression of Cyclooxygenase-2, but not of Cyclooxygenase-1 in Cultured VSMC

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NO and cyclooxygenase-2 (COX-2) are contributes to vascular inflammation induced by various stimulation. The mechanism, which explains a linkage between NO and COX-2, could be of importance in promoting pathophysiological conditions of vessel. We investigated the effects of NO donors on the COX-1 and COX-2 mRNA/protein expression, as well as the nitrite production in culture medium of vascular smooth muscle cell (VSMC). VSMC was primarily cultured from thoracic aorta of rat. In this experiments, COX-1 and COX-2 mRNA/protein expressions were analysed and nitrite productions were investigated using Griess reagent. VSMC did not express COX-2 protein in basal condition (Non-lipopolysaccharide (LPS) stimulated). In LPS-stimulated experiments, after 3 hours of NO donor pretreatment, LPS 10 μg/ml was treated for 24 hours. COX-1 protein expressions were unchanged by SNP and NOR-3. NOR-3 significantly increased COX-2 mRNA/protein expression under LPS stimulation. In contrast, SNP did not increase COX-2 mRNA/protein expression under LPS stimulation. Nitrite production was higher in NOR-3 treatment than SNP treatment under LPS stimulation. These results suggest that the expression of COX-2 in VSMC is regulated by NOR-3, COX-2 expressions were depending on the types of NO donor and LPS stimulation in VSMC.

Key Words: COX-2, SNAP, SNP, NOR-3, VSMC

INTRODUCTION

Nitric Oxide (NO) exhibits a broad spectrum of biological effects, including modulation of vascular tone, neurotransmission, hormone release, and cell growth. In addition, NO has a multifunctional role in inflammation (Choi et al, 2001) and various proinflammatory effects of NO have been described, including an increase in vascular permeability, cytotoxicity and tissue damage (Thimmerman & Vane, 1990; Szabo et al, 1993).

In the VSMC, COX-2 dependent mechanism was required for inflammation in response to various stimuli including LPS (Park et al, 2006). This mechanism might contribute to vascular injury or pathophysiological conditions in which elevated levels of cytokines are evident.

The two main isoenzymes of cyclooxygenase, COX-1 and COX-2, are differently regulated. COX-1 is constitutively expressed in numerous tissue types and its expression is, in general, not regulated. In contrast, COX-2 is typically undetectable in most tissues under normal physiological condition but can be rapidly expressed at high levels following growth factors and bacterial endotoxin stimulation (Smith et al, 1996; Williams & DuBois, 1996). Both COX isozymes are found in most tissues.

Furthermore, an interaction between NO and COX-2 has been suggested to be present in the various tissues (Chwalisz & Garfield, 1998; Yang et al, 2006). NO donors, such as isosorbide mononitrate (IMN) and nitroglycerin, have been documented to induce cervical ripening in pregnant women through COX-2 activation (Thomson et al, 1997; Ekerhovd et al, 2003). Besides, COX-2 induction in VSMC down-regulated NO accumulation through thromboxane A_2 production (Takahiro et al, 2002).

Such a linkage between two proinflammatory substances could be of importance in promoting vascular disease. In addition, inflammatory response can induce nitric oxide synthase (NOS) and COX-2. Given the general similarities between the effects of NOS and COX-2, an interaction of the two systems might be necessary for inflammatory response of VSMC.

In this study, we investigated to clarify, an NO donor, S-nitros-N-acetylpenicillamine (SNAP), sodium nitroprusside (SNP) and NOR-3 could affect COX-1 and COX-2 expressions in cultured VSMC treated with/without LPS. Therefore, we determined whether NO exerted a direct stimulatory effect on COX-2 expression and to investigated

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ABBREVIATIONS: COX-2, cyclooxygenase-2; VSMC, vascular smooth muscle cell; NO, nitric oxide.

the intracellular signalling mechanisms underlying this interaction in VSMC.

METHODS

Cell culture

Rat aortic VSMC was derived from thoracic aorta of adult male Sprague Dawley rat by primary explant culture techniques. Briefly, excised thoracic aortas were minced into small pieces and washed by HBSS (Sigma, St. Louis, USA). These pieces were plated onto a culture dish containing 50% fetal bovine serum (FBS) (Gibco BRL, Grand Island, USA) and antibiotics-antimycotics (penicillin 100 U/ml, amphotericin 2.5 μg/ml and streptomycin 100 μg/ml) in DMEM (Sigma, St. Louis, USA). Outgrowthed cells were plated and grown in DMEM supplemented with 10% FBS and antibiotics-antimycotics. Cells were maintained in a humidified 95% O_2-5% CO_2 incubator. Media were changed two or three times a week.

Western blot

Confluent VSMC grown in media supplemented with 10% FBS were prepared. After drug treatment, cells were washed with phosphate buffered saline (PBS) and harvested by scraping. Protein extracts (12,000$\times$g supernatant) in the lysis buffer (20 mM Tris, pH 7.5, EDTA 0.5 mM, EGTA 0.5 mM, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μl/ml β-mercaptoethanol) were separated by electrophoresis (80 μg protein per lane) on 10% polyacrylamide gels in the presence of sodium dodecylsulphonate and then transferred onto nitrocellulose membranes (Protran, Schleicher & Schuell, Germany). The loading and transfer of equal amounts of protein in each lane was verified by staining the protein bands with Ponceau S (0.2% in 3% trichloroacetic acid). After extensive washing with distilled water to remove the protein stain, blots were blocked with 5% non-fat dry milk in PBS. The immobilized protein was visualized by subsequent incubation with a primary antibody (COX-1, COX-2, NFκB p65, pNFκB p65 antibody) according to each experiments and a secondary polyclonal peroxidase-conjugated anti-rabbit antibody followed by staining with the enhanced chemiluminescence (ECL) technique developed by NEN life science.

Measurement of nitrite production

Nitrite contents were measured by using the Griess reagent/reaction. In brief, VSMC were incubated with LPS (O26:B6, Sigma, St. Louis, USA) and/or NO donors. The culture media supernatants were recovered; after centrifugation (12,000 g, 5 min) nitrite accumulation was measured by mixing equal volumes of supernatants and the Griess reagent [0.1% N-(1-naphtyl)ethylenediamine dihydrochloride and 1% sulfanilamide in 2% H_3PO_4]. Nitrite concentrations were determined at an optical density of 550 nm by comparison with standard solutions of sodium nitrite prepared in the same culture media.

Real-time reverse transcription polymerase chain reaction

Total cellular RNA was isolated using TRIzol reagent (Invitrogen Co, California, USA). RNA concentrations were calculated from absorbance at 260 and 280 nm by nano drop (NanoDrop Technologies Inc, Wilmington, USA). For reverse transcriptation, the following components were added to the reaction vials: 1 μg of total RNA, $MgCl_2$, 10$\times$ RT buffer, RNase inhibitor, dNTPs, AMV-RT in a total volume of 10 μl. The vials were incubated for 60 min at 42°C, thereafter the reverse transcriptation was terminated by heating at 95°C for 5 min.

The assay for COX-2 mRNA expression was set up using the Light Cycler system (Wittwer et al, 1997) with SYBR Green I sequence non-specific detection (Roche Diagnostics, Swiss). After an initial Taq activation at 95°C for 10 min, Light Cycler PCR was performed using 40~45 cycles with the following cycling conditions: 95°C for 10 s, 60°C for 5 s, 72°C for 14 s. Directly after the PCR, the machine performed a melting curve analysis by slowly (0.1°C/s) increasing the temperature from 65 to 95°C, with a continuous registration of changes in fluorescent emission intensity. Each PCR was performed in a total volume of 10 μl, made from diluted 100 ng cDNA, forward and reverse primers (0.4 μmol each), and SYBR Green I master mix (2.2 μl, including Taq polymerase, reaction buffer, $MgCl_2$, SYBR Green I dye, and dNTP mix (with dUTP instead of dTTP). The sequence of the two COX-2-specific primers was 5-GCAAATCCTTGCTGTTCCAATC-3 (forward) and 5-GGAGAAGGCTTCCCAGCTTTTG-3 (reverse), and the predominant cDNA amplification product was predicted to be 335 bp in length. The sequence of the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)-specific primers was 5-GTCATGAGCCCTTCCACGATGC-3 (forward) and 5-AATCTACTGGCGTCTTTCACC-3 (reverse), and predominant cDNA amplification product was predicted to be 300 bp in length.

Statistical analysis

Results are reported as mean$\pm$S.E.M. Comparisons of the means of the two groups were performed by the paired t test. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

Effects of NO donors on COX-1 and COX-2 protein expression

To examine the effects of NO donors on the LPS-stimulated expression of COX-2 protein, VSMC was pretreated with SNP or NOR-3 for 3 hours, then activated with 10 μg/ml LPS for 24 hours. There were an increase in the density of bands on immunoblots, corresponding to COX-2 protein after LPS stimulation, whereas COX-1 protein expressions were not changed. In VSMC, NOR-3 dose-dependently increased LPS-stimulated COX-2 protein expression. Otherwise, SNP revealed inhibitory effects on LPS-stimulated COX-2 protein expression. GAPDH levels remained unchanged during incubation with SNP or NOR-3 (Fig. 1).

Effects of NO donors on nitrite production

To investigate the NO releasing effects of SNP or NOR-3 in the LPS-stimulated VSMC, nitrite assay was done. Drug

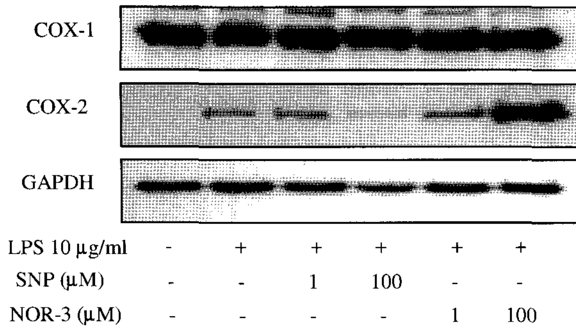

Fig. 1. The Effects of NO donors on LPS induced COX-1 and COX-2 protein expression. Western blot analysis of COX-1 and COX-2 protein, and house keeping gene GAPDH. VSMC was pretreated with SNP or NOR-3 for 3 hours, then activated with 10 μg/ml LPS for 24 hours. LPS: Lipopolysaccharide, SNP: Sodium nitroprusside.

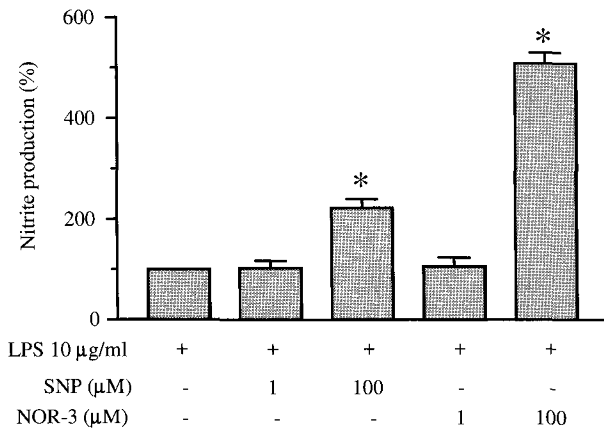

Fig. 2. The effects of NO donors on LPS induced nitrite production. VSMC was pretreated with SNP or NOR-3 for 3 hours, then activated with 10 μg/ml LPS for 24 hours. Values represent Mean±S.E.M. (n=6 for each group). *$p < 0.05$: significantly different from LPS 10 μg/ml. LPS: Lipopolysaccharide, SNP: Sodium nitroprusside.

treatment protocol was the same as above mentioned. NOR-3 100 μM increased nitrite production potently in LPS-stimulated VSMC. When VSMC were incubated with SNP 100 μM, nitrite production was increased but the change was smaller than NOR-3 100 μM (Fig. 2).

Effects of NO donors on COX-1 and COX-2 mRNA expression

As shown in Fig. 3, increased COX-2 mRNA expressions by SNAP, SNP and NOR-3 were noted in real-time PCR experiment. Among 3 types of NO donors, NOR-3 most potently increased COX-2 mRNA expression (Fig. 3).

We also examined the effects of SNAP, SNP and NOR-3 on the LPS-stimulated expression of COX-2 mRNA using real-time PCR. VSMC was pretreated with SNAP, SNP or NOR-3 for 3 hours, then activated with 10 μg/ml LPS for 24 hours. In VSMC, NOR-3 increased LPS-stimulated COX-2 mRNA expression, whereas, SNAP and SNP did not induce alterations of COX-2 mRNA expressions (Fig. 4).

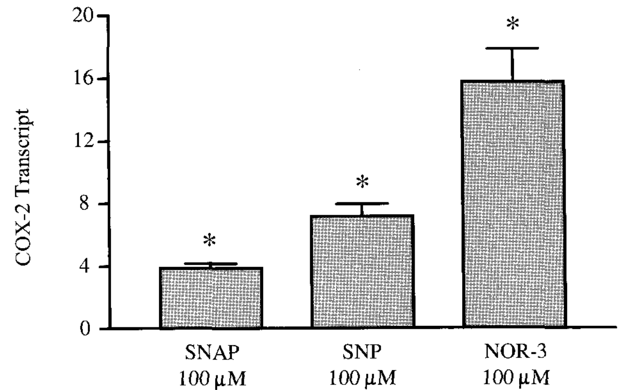

Fig. 3. The effects of NO donors on COX-2 mRNA expression. Real-time PCR analysis. VSMC was treated with SNAP, SNP or NOR-3 for 24 hours. Values represent Mean±S.E.M. (n=3 for each group) *$p < 0.05$: significantly different from non-treated. SNAP: S-nitros-N-acetylpenicillamine, SNP: Sodium nitroprusside.

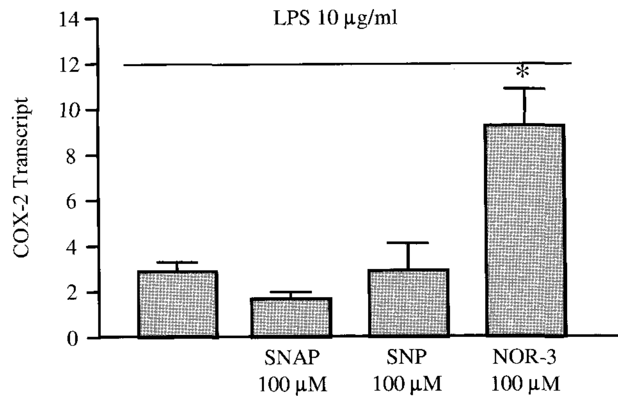

Fig. 4. The effects of NO donors on LPS induced COX-2 mRNA expression. Real-time PCR analysis. VSMC was pretreated with SNAP, SNP or NOR-3 for 3 hours, then activated with 10 μg/ml LPS for 24 hours. Values represent Mean±S.E.M. (n=3 for each group) (n=3 for each group). *$p < 0.05$: significantly different from LPS 10 μg/ml. LPS: Lipopolysaccharide, SNAP: S-nitros-N-acetylpenicillamine, SNP: Sodium nitroprusside.

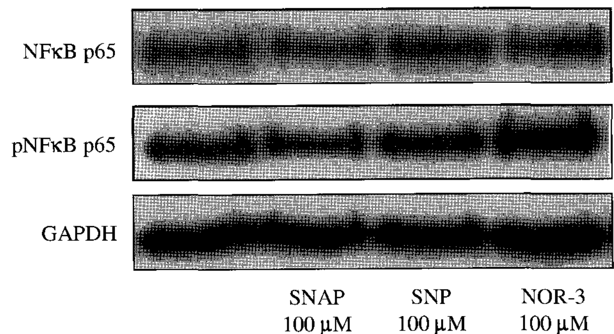

Fig. 5. The effects of NO donors on NFκB p65 and phosphorylated NFκB p65 expression. Western blot analysis of NFκB p65 and phosphorylated NFκB p65, and house keeping gene, GAPDH. VSMC was treated with SNAP, SNP or NOR-3 for 24 hours. SNAP: S-nitros-N-acetylpenicillamine, SNP: Sodium nitroprusside.

Effects of NO donors on NFκB p65 and pNFκB p65 expression

To examine the effects of SNAP, SNP and NOR-3 on the inflammatory signal transduction, phosphorylations of NFκB p65 were studied with VSMC treated with SNAP, SNP or NOR-3. As shown in Fig. 5, NOR-3 increased phosphrylation of NFκB in VSMC. Otherwise, SNAP and SNP did not induce alterations in phosphrylation of NFκB in VSMC (Fig. 5).

DISCUSSION

The present study was undertaken to examine the role and mechanism of NO in regulation of COX-2 expression in cultured VSMC. There is a growing concern and indeed evidence showing that premature mortality due to the cardiovascular disease is promoted by vascular inflammatory mechanisms (Festa & Haffner, 2005). After vascular inflammation, such as LPS stimulation, diverse signalling mechanisms became activated, leading to COX-2 expression. We previously showed that the modulation of LPS signalling is important in VSMC proliferation and survival in septic shock (Choi et al, 2001). Increment of iNOS and COX-2 expression is found in the several vascular inflammatory disease (Niederberger et al, 2004; Nachtigal et al, 2005). The NFκB activation, in particular, has recently received interest in regard to its role in NO induced vascular inflammation (Ahn et al, 2005; Vo et al, 2005).

COX-2 is the inducible isoform of cyclooxygenase whose expression is initiated by inflammatory process (Vane, 1994). COX-2 is expressed in many cell types and has a variety of different signal transduction roles. In addition to its role in the inflammation process, it has also been shown to regulate cell proliferation in many cell types (Cao & Prescott, 2004; Hasegawa et al, 2005).

NO is a free radical produced endogenously by both constitutive and inducible NO synthase isozymes (Nathan & Xie, 1994; Rodeberg et al, 1995). It is a relatively stable, small, lipid-soluble gas that can pass easily from cell to cell and is an important mediator of many biological effects. NO synthesized from L-arginine by NO synthases, has been identified to play an important role in cell communication, cell defense, and cell injury. During vascular inflammation, VSMC are the major source and target of NO (Choi & Lee, 2004). NO has been demonstrated to trigger the expression of proinflammatory and protective gene products in kidney (Pfeilschifter, 2002).

Induction of COX-2 has been suggested to promote cell growth, inhibit apoptosis, and enhance cell motility and adhesion. COX-2 is induced by a number of inflammatory, mitogenic, and physical stimuli.

Some studies have shown that NO was capable of stimulating the activity of COX, both in vitro and in intact cells, whereas others have found no effect of NO on the activity of COX. It has also been reported that tetrahydrobiopterin (BH_4), which serves as a cofactor for optimal activity of iNOS, could play a modulatory role in the expression of COX-2 in human mesangial cells (Perez-sala et al, 1997). However, little is known in the literature about the mechanism of LPS triggered NO production and COX-2 expression, and its role in vascular inflammation.

We demonstrated that NOR-3 potentiated an increment of COX-2 mRNA and protein expression induced by LPS in VSMC. This finding indicates that an interaction between NO and prostaglandin synthesis is present in the vascular inflammation. Several studies have demonstrated that NO can induce the expression of COX-2 in various tissues, thereby increasing the release of endogenous prostaglandin (Salvemini et al, 1994; Davidge et al, 1995). Although, SNP led to significant increase in nitrite production, SNP did not increase COX-2 mRNA and protein levels under LPS stimulation, this needed to be illucidated.

With regard to a possible spontaneous source of COX-2 expression, we were unable to detect any expression of COX-2 protein without LPS stimulation. However, COX-2 mRNA expression was detected without LPS stimulation using Real-Time PCR experiment. The possible cause of the up-regulatory effect of NO on COX-2 expression under LPS stimulation might be phosphorylation of NFκB. NFκB is known to be indispensable for LPS induced expression of COX-2 (Shimomura-Shimizu et al, 2005).

On the other hand, D'Acquisto et al. (2001) have reported that the inhibitory effect of NO on inducible cyclooxygenase expression in J744 macrophage is due to blocking the activation of NFκB and nuclear factor interleukin 6. They also indicated that the inhibition of prostaglandin E_2 production by NO was not dependent on direct inhibition of COX-2 activity and suggested that the inhibition might be at the transcriptional level. Similarly, nitroflurbiprofen, a NO releasing derivative of anti-inflammatory drug, flurbiprofen, also significantly inhibited prostaglandin E_2 production by LPS activated microglia (Ajmone-Cat et al, 2001).

In conclusion, the current data demonstrate that the expression of COX-2 in VSMC is regulated by NOR-3, COX-2 expressions were depending on the types of NO donor and LPS stimulation in VSMC. In addition, the down-regulation of COX-2 expression induced by SNP was might be through inhibition of same pathway. This finding suggest the idea that COX-2 expression in VSMC is dependent on types of NO donor, and this may be especially important when inflammation is evident.

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