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Roles of Purine Nucleotides and Adenosine in Enhancing NOS II Gene Expression in Interleukin-1β-Stimulated Rat Vascular Smooth Muscle Cells

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The production of nitric oxide (NO) by vascular smooth muscle cells (VSMC) is stimulated by interleukin-1β (IL-1β). This is enhanced in a dose-dependent manner by ADP, although it alone failed to induce nitrite accumulation. Purine nucleotides and their nonhydrolizable analogues as well as adenosine also exhibit variable enhancing effects. This enhanced nitrite formation was due to induction of the NO synthase (NOS II) gene as judged by Northern hybridization using an NOS II specific probe and by Ca^{2+} independency of the NOS activity. 8-(*p*-Sulfophenyl)-theophylline, a blocker of adenosine receptors, suppressed the enhanced NO production by adenosine and ADP to the level of that with IL-1β alone. These data indicate that activation of the adenosine receptor on VSMC may enhance production of NOS II by modulating a signal transducing pathway of IL-1β. Although cAMP is a candidate as the second messenger, it was not significantly elevated by either ADP or adenosine treatment in IL-1β-stimulated cells. This mechanism might be stimulated under conditions with release of various purine and their derivatives.

Keywords: Nitric oxide, nitric oxide synthase, vasodilatation, purino receptor, adenosine receptor

Abbreviations: NO, nitric oxide; NOS, NO synthase; VSMC, vascular smooth muscle cells; IL-1β, interleukin-1β; TNF, tumor necrosis factor; bFGF, basic fibroblast growth factor; TGF-β, transforming growth factor-β; PDGF, platelet-derived growth factor; LPS, lipopolysaccharide; AMP-PNP, β, γ-imidoadenosine 5′-triphosphate; AMP-PCP, β, γ-methyleneadenosine 5′-triphosphate; AMP-CPP, α, β-methyleneadenosine 5′-triphosphate.

It is well known that many tissues, such as the endothelium, macrophages, liver and brain, produce nitric oxide (NO) by an NADPH-dependent NO synthase (NOS) using L-arginine and molecular oxygen as substrates.[1] Three NOS isozymes have been identified and cloned.[2] One form, NOS I, is constitutively expressed mainly in the brain and its activity is Ca^{2+} dependent.[3] The second form (NOS II) is induced in macrophages,[4] hepatocytes,[5] and smooth muscle cells[6,7] by stimulation with agents such as cytokines and lipopolysaccharide (LPS) and is Ca^{2+}-independent. Another form, NOS III, that is membrane

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associated was originally found in endothelial cells and its activity is Ca^{2+} dependent.[8]

NO plays a pivotal role in cardiovascular functions.[9] In endothelial cells it activates cytosolic guanylate cyclase of adjacent vascular smooth muscle cells (VSMC) by a paracrine mechanism. Produced cGMP leads to VSMC relaxation and results in vasodilation. VSMC themselves also produce NO in response to cAMP-elevating agents[10] and to cytokines such as interleukin-1β (IL-1β), tumor necrosis factor (TNF),[6] and basic fibroblast growth factor (bFGF).[11] However, NO production is suppressed by transforming growth factor-β (TGF-β).[12,13] Several vasoactive factors such as angiotensin II,[14] thrombin,[15] platelet-derived growth factor (PDGF),[13,16] and insulin-like growth factor 1[17] are also known to suppress induction of NOS II, whereas plasmin can induce its gene expression.[18]

Adenine nucleotides, which can be easily converted to adenosine, are thought to be released upon necrosis of atherosclerotic plaques[19] and from injured endothelial cells after balloon cathetelization.[20,21] Purine nucleotides are known to stimulate vascular endothelial cells to release NO by elevating intracellular Ca^{2+} with resultant vasodilatation. Moreover, adenosine is also known to induce vasodilatorial effects following binding to adenosine receptors.[22] In this report, we demonstrate that various nucleotides and adenosine augment production of NO in VSMC whose NOS II gene expression had been stimulated with IL-1β.

EXPERIMENTAL

Methods

L-[U-^{14}C]Arginine monohydrochloride (319 mCi/mmol) was obtained from Amersham Corp. Leupeptin, pepstatin, and calmodulin were Sigma products. NADPH and tetrahydrobiopterin were from Boehringer Mannheim and Research Biochemical Inc., respectively. (*p*-Amidinophenyl) methanesulfonylfluoride and FAD were obtained from Wako Pure Chemical Industries and Dowex 50W-X8 from Muromachi Kagaku Kogyo. Recombinant human IL-1β was kindly provided by Otsuka Pharmaceutical Co. LTD. The other reagents were of the highest grade available.

Isolation and Culture of VSMC from Rat

VSMC were isolated from free-floating explants of rat aorta as essentially described[23] in accord with the ethical standards of the Helsinki Declaration of 1975. Briefly, aorta was dissected from rats and incubated in Dulbecco's modified Eagle's medium containing 100 units of both penicillin and streptomycin and supplemented with 20% heat-inactivated fetal bovine serum (GIBCO/BRL) at 37°C under an atmosphere of 95% air and 5% CO_2. The explants were cut longitudinally, and then the endothelial cells were removed. The isolated medial membrane was cut into small pieces and incubated for a day. Following supplementation with fresh medium, the tissue was again incubated for a few days. VSMC were removed by trypsinization and maintained in the same medium.

Assay of NOS

Following stimulation with IL-1β, the cells were collected and sonicated in 50 mM Tris-HCl (pH 7.4) buffer containing 250 mM sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, 20 μM (*p*-amidinophenyl) methanesulfonylfluoride, 5 μM leupeptin, and 2 μM pepstatin. Nitric oxide synthase activity was determined as the conversion of radiolabelled L-[U-^{14}C]arginine to L-citrulline by a minor modification[24] of the method described previously.[25] Briefly, 10 μl of the sonicate was incubated for 10 min at 37°C in 50 mM Hepes buffer (pH 7.8) containing 1 mM DTT, 1 mM $CaCl_2$, 0.1 mM tetrahydrobiopterin, 1 mM NADPH, 10 μg/ml calmodulin 10 μM FAD, and 1.55 μM L-[U-^{14}C] arginine, in a final volume of 100 μl. The reaction was terminated by the addi-

tion of 200 µl of buffer A (100 mM Hepes and 10 mM EDTA, pH 5.2). The whole reaction mixture was then applied to a 300 µl Dowex 50W-X8 column (Na^+ form, 200–400 mesh) that had been equilibrated with buffer A. Citrulline was eluted with 0.5 ml of buffer A and then its radioactivity was determined with a liquid scintillation counter.

cDNA Probes

An 818 bp-NOS II cDNA fragment synthesized from total RNA of RAW264.7 mouse macrophage cells stimulated with 10 ng/ml LPS was used as described previously.[26] Rat β-actin cDNA was used as an internal control.

RNA Preparation and Northern Blotting

Total RNA was prepared from VSMC culture in 10 cm dishes essentially as described,[27] and quantitated by measuring the absorbance at 260 nm. Twenty µg of total RNA were heat-denatured at 65°C for 15 min in the presence of 50% formamide and the running gel buffer (40 mM Mops, 10 mM sodium acetate, and 1 mM EDTA, pH 7.0), and then electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. The size-fractionated RNAs were transferred onto a Zeta-Probe membrane (Bio-Rad) for 20 to 40 h by capillary action and the blotted RNAs were immobilized on the membrane by UV irradiation and incubation for 2 h at 80°C under vacuum. After hybridization with a ^{32}P-labeled NOS II cDNA probe at 42°C in the presence of 50% formamide, the membranes were washed twice at 55°C with 2 × SSC (1 × SSC; 15 mM sodium citrate and 150 mM NaCl, pH 7.5) and 0.1% SDS for 80 min, and then twice at high stringency in 0.3 × SSC and 0.1% SDS for 60 min at 65°C. Kodak XAR films were exposed for 1–3 days with an intensifying screen at −80°C. The blots were stripped by boiling in 0.1% SDS and 0.1 × SSC and rehybridized with a β-actin cDNA probe using conditions given above.

Quantification of Nitrite Formed by VSMC

The cells (1×10^5 cells per well in 12-well dishes) were incubated for 24 h in the culture medium supplemented with 5 ng/ml IL-1β in the presence of various concentrations of given reagents. Following incubation and centrifugation the nitrite in the supernatant was quantitated as described[28] using the Griess reagent.[29]

cAMP Assay

The cells (1.5×10^5 cells per well in 12 well-dishes) were incubated with various agents for 30 min. The reaction was terminated by adding 0.5 ml of ice cold 0.1 N HCl. After centrifugation at 15000 rpm for 10 min, the cAMP content of 0.1 ml of the supernatant was determined by radioimmunoassay using a cAMP assay kit (Yamasa).

Statistical Analysis

The paired Student's *t* test was used to compare the significance of the differences between data. All data are expressed as means ± SD.

RESULTS

ADP Enhanced Nitrite Formation by IL-1β-Stimulated VSMC

Since VSMC have the ability to produce NO in response to various stimuli, we evaluated the effects of ADP on NO formation by measuring levels of its nitrite, a spontaneous oxidation form of NO, in the medium (Fig. 1). The level of nitrite was augmented by the presence of IL-1β alone as previously reported[6] and was enhanced by addition of ADP to the medium (Fig. 1B). ADP alone failed to stimulate nitrite production. Levels of nitrite measured at 24 h after ADP addition increased in a dose-dependent manner, but was not saturated even at 1 mM ADP (Fig. 1C). The effective concentration of ADP in the

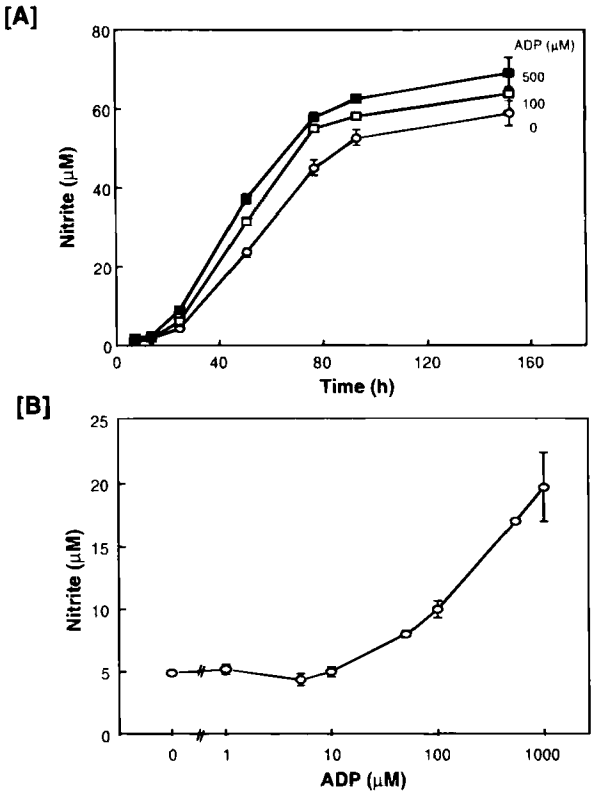

FIGURE 1 Effect of IL-1β and ADP on the release of nitrite by VSMC. **(A)** Time course of nitrite accumulation in the VSMC culture media in the presence of 5 ng/ml IL-1β and 0 (○), 100 (□), and 500 (■) μM ADP. **(B)** Dose-response curve of ADP in the presence of 5 ng/ml IL-1β.

medium, however, would be lower because ADP is gradually hydrolyzed to adenosine during incubation by exo-nucleotidases of the VSMC.[30]

The Effect of Various Nucleotides on Nitrite Formation

To investigate whether the enhancement of NO production by VSMC was specific for ADP, we examined the stimulatory effects of various nucleotides and related compounds on nitrite formation. As shown in Table I, AMP-PNP and AMP-PCP, which have a nonhydrolizable bond between the β and γ phosphate, were effective compounds, eventhough they can be converted to AMP and adenosine by exonucleotidases. Adenine nucleotides, GTP, and adenosine seemed to have similar stimulatory effects on nitrite formation whereas AMP-CPP, CTP, and UTP were less effective.

ADP Enhanced NOS II Gene Transcription by IL-1β

The stimulatory effect of purine on NO release from endothelial cells is attributed to activation of NOS III via increased intracellular Ca^{2+}, but not to the induction of gene expression.[9] Since ADP alone had no effect on NO release by VSMC, we examined the NOS II gene expression in VSMC stimulated with IL-1β by Northern blot analysis, in the presence or absence of ADP. NOS II mRNA, which was initially below the detection level, was induced by IL-1β and its expression

TABLE I Effects of various nucleotides on nitrite production induced by IL-1β in VSMC.

	Medium nitrite, μM (%)	
Nucleotides	100 μM	500 μM
IL-1β (5ng/ml)		
alone	2.7 ± 1.0 (100)	2.7 ± 1.0 (100)
+AMP	5.1 ± 0.7 (188)	9.6 ± 0.7 (355)
+ADP	7.4 ± 0.4 (274)	8.9 ± 1.2 (329)
+ATP	6.7 ± 2.2 (248)	12.1 ± 0.6 (448)
+AMP-CPP	5.5 ± 0.3 (203)	4.8 ± 0.2 (177)
+AMP-PCP	9.2 ± 0.4 (340)	21.6 ± 2.4 (800)
+AMP-PNP	11.7 ± 0.7 (433)	17.2 ± 0.4 (637)
+Adenosine	8.4 ± 0.3 (311)	11.3 ± 1.2 (418)
+CTP	4.2 ± 0.8 (155)	5.4 ± 0.5 (200)
+GTP	4.7 ± 0.7 (174)	11.3 ± 0.8 (418)
+UTP	3.6 ± 1.0 (133)	7.1 ± 1.5 (262)

[a]percent nitrite compared to IL-1β alone.
Cells (5×10^5/ml) were plated and cultured for overnight and then various nucleotides were added at two concentrations. After 24 h incubation, amounts of nitrite in the culture media were determined in triplicate. Data are presented as mean ± S.D. of triplicate experiments.

was enhanced by ADP (Fig. 2). The induction of the mRNA was dependent on the concentrations of ADP and roughly consistent with the levels of nitrite produced. The mRNA levels of NOS II in VSMC stimulated with IL-1β in the presence of various other nucleotides were also elevated (data not shown).

To confirm that nitrite accumulation following administration of ADP was due to the induced NOS II isoform, we measured the NOS activity in the cytosolic fraction of VSMC both in the presence and absence of Ca^{2+} (Table II). NOS activity was negligible in unstimulated cells, but a significant amount was detected after IL-1β-stimulation even in the absence of Ca^{2+}. This indicates that enhanced NO formation by ADP could be attributed to the expression of NOS II. When the effect of cycloheximide on the enhanced expression of NOS II by adenosine was examined, cycloheximide itself increased the NOS II mRNA (Fig. 3) probably due to prevention of mRNA breakdown as reported,[30] indicating that the induction of NOS II mRNA by adenosine in IL-1β-stimulated VSMC was independent of new protein synthesis. Then we examined possible involvement of NF-κB, which exists constitu-

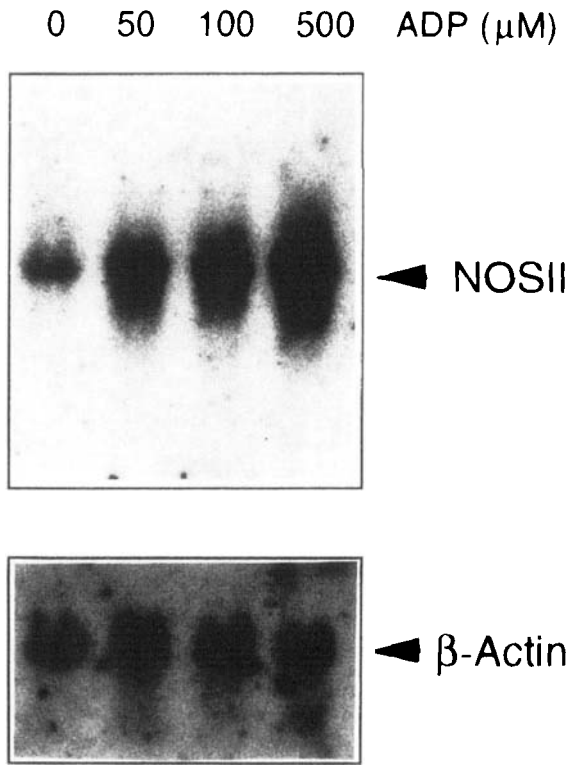

FIGURE 2 Northern blot analysis of NOS II mRNA expression in VSMC. VSMC was stimulated with 5 ng/ml IL-1β and varying concentration of ADP. Twenty μg of total RNA prepared from the VSMC were analyzed by hybridization with mouse NOS II cDNA **(A)** and rat β-actin cDNA **(B)** as probes.

TABLE II Effect of ADP on NOS activities in the presence or absence of Ca^{2+}

	NOS activity (pmol/min/mg)	
	100 μM $CaCl_2$	1 mM EDTA
Control	0.8 ± 1.4	0.2 ± 0.4
IL-1β	5.2 ± 2.2	4.0 ± 1.5
ADP	0	0.1 ± 0.2
IL-1β + ADP	11.6 ± 3.7	9.7 ± 3.4*

*$p < 0.05$ compared to IL-1β alone.
After incubation with IL-1β (5 ng/ml) in the presence or absence of ADP (500 μM), NOS activities of cell lysate were measured in triplicate. Data are presented as mean ± S. D. of triplicate experiments.

tively in cytoplasm as an inactive form and whose activation is essential for the NOS II induction,[31] by gel shift assay using both upstream and downstream sequences corresponding to two NF-κB sites located at 5'upstream region of mouse NOS II gene, but did not see change in DNA binding capacity of nuclear extracts by adenosine (data not shown).

Involvement of an Adenosine Receptor in the Enhanced NOS II Gene Expression

Since ADP can be hydrolyzed to adenosine, a strong vasodilator,[32] we investigated the possible participation of an adenosine receptor in the induction of NOS II gene. 8-(*p* Sulfophenyl)theophylline, an adenosine receptor blocker,[22] at

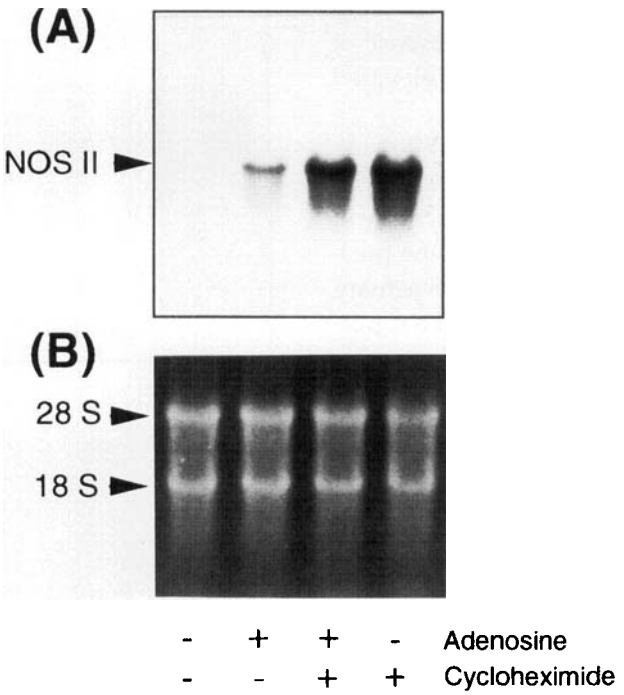

FIGURE 3 Effects of cycloheximide on NOS II mRNA expression in IL-1β-stimulated VSMC by adenosine. **(A)** Total RNA were prepared from the VSMC stimulated with 10 ng/ml IL-1β in the presence of 500 μM adenosine and/or 50 μM cycloheximide as indicated. Twenty μg total RNA were analyzed by Northern blotting as in Figure 2. **(B)** Positions of 18 S and 28 S ribosomal RNA were indicated.

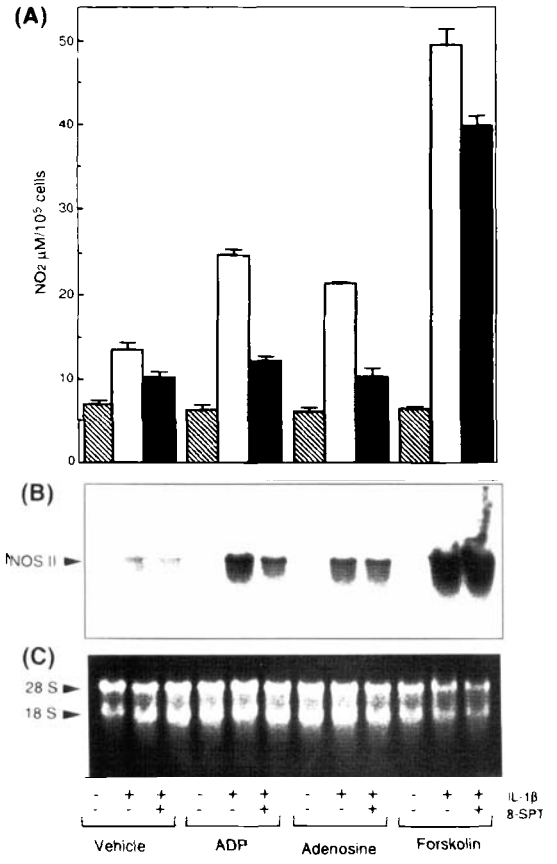

FIGURE 4 Effect of an adenosine receptor blocker (8-SPT) on the nitrite formation and NOS II mRNA expression enhanced by ADP and adenosine. VSMC were cultured in the presence of 10 ng/ml IL-1β and 500 μM ADP, adenosine, and AMP-PNP with or without 100 μM 8-SPT. After 24 h, nitrite in the medium **(A)** and NOS II mRNA levels **(B)** of these cells were determined. **(C)** Positions of 18 S and 28 S ribosomal RNA were indicated.

100 μM totally inhibited the enhancing effect of adenosine on nitrite formation and on NOS II mRNA expression as well as those of ADP, and part of forskolin (Fig. 4). This suggested that ADP-stimulated NOS II induction operates through an adenosine receptor mechanism. We then measured the intracellular levels of cAMP, a possible second messenger for adenosine receptor-mediated signaling.[22] As shown in Fig. 5, while forskolin elevated the levels of cAMP as reported,[20] neither ADP nor adenosine affected them in VSMC stimulated with IL-1β. Thus cAMP was not likely mediating the signal of these agents in NOS II gene expression in VSMC.

DISCUSSION

NO plays a pivotal role in the cardiovascular system in regulating blood flow by its relaxation effects through activation of guanylate cyclase in VSMC. It is well known that NOS III of endothelial cells constitutively produce NO and the production is augmented by physiological stimulators such as thrombin, bradykinine, substance P, and shear stress.[9] The effects of these substances, however, are due to activation of NOS III by increasing intracellular Ca^{2+} and not through gene induction. VSMC, on the other hand, express NOS II, which is encoded by the same gene as that induced in macrophages and hepatocytes.[7] The regulation of gene expression, however, seems to vary for different cell types. IFN-γ and LPS are the most potent stimulators for rodent macrophages, while IL-1β and TNFα are more effective on VSMC.

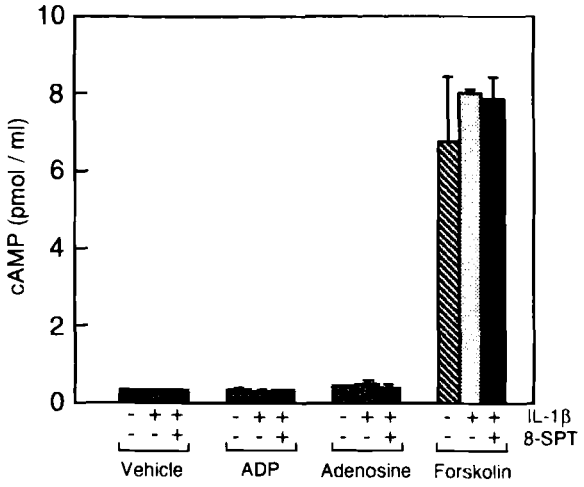

FIGURE 5 Effect of ADP, adenosine, and 8-SPT on the levels of cAMP. VSMC were stimulated under the same conditions as described in Figure 3 legend. Intracellular cAMP levels were determined at 30 min after addition of agents indicated by radioimmuno assay.

We had shown that purine nucleotides and adenosine potentiate NOS II induction in VSMC by IL-1β, but ADP alone does not induce gene expression (Figs. 1 and 2, Table I). Its stimulatory effect is mainly mediated by a purino receptor and adenosine receptors as judged by its ligand specificity and the blocking effect of 8-(*p*-sulfophenyl)theophylline (Fig. 4). These data indicate that binding of extracellular nucleotides to the above receptors may enhance production of NOS II by modulating a signal transducing pathway of IL-1β. However, this stimulatory effect of ADP was not sufficient for NOS II gene induction because without IL-1β it had no effect. Since cAMP-elevating agents are known to induce NOS II in VSMC[10] and cAMP is one of the intracellular signals of these receptors in VSMC,[22] the enhancing effect of such compounds may be mediated by cAMP. However, our data did not support the involvement of cAMP in this adenosine receptor-mediated NOS II induction (Fig. 5). Although involvement of cAMP-dependent protein kinase in this receptor-mediated effect is hypothesized, no cAMP-responsive element is found in the 5′ upstream region of the NOS II gene.[33]

NO binds to the heme of guanylate cyclase and stimulates production of cGMP, which then brings about smooth muscle relaxation, resulting in vasodilatation.[1] Because stimulation of the purino and adenosine receptors enhanced induction of NOS II gene expression in VSMC, a part of the vasodilatatory effect of the stimulation of these receptors may be mediated by NO. Under inflammatory conditions, such as endothelial injury and balloon cathetelization,[20,21] activated immune response cells invade to smooth muscle layers and produce inflammatory cytokines such as IL-1β, TNF, and IFN-γ. These agents are also able to induce NOS II in VSMC and in damaging adjacent cells release nucleotides into the plasma.[34] The presence of ADP enhances gene expression by these cytokines, which would result in NO production and relaxation of VSMC. Since the fibrinolytic enzyme, plasmin, is known to enhance NO release from VSMC,[18] ADP in conjunction with plasmin may function in VSMC as a protective factor by maintaining blood flow through the induction of NOS II during formation of a thrombus.

Adversely, chronic inflammation produces a large amounts of NO, which can rapidly react with superoxide to produce a toxic intermediate peroxynitrite[35] and thus cause serious endometrial and incite the development of damage leading to life-threatening diseases such as atherosclerosis. Moreover, NO may also lower antioxidant protective mechanisms by inactivating glutathione peroxidase[36] and by decreasing the levels of superoxide dismutases[37] and modify the cellular redox state. Since ATP also releases NO from macrophages,[38] such a continuous production of NO would be harmful to tissues. In support of this, extensive nitration of protein tyrosine residues is found in human arteries of patients with atherosclerosis.[39] It is speculated that NO released from VSMC induces their death with the consequent release of bFGF and stimulation of proliferation of adjacent endothelial cells.[40] In summation, adenosine and adenine nucleotides released from dead VSMC would stimulate NO production in surrounding cells and accelerate the release of bFGF, and thus participate in neovascularization of atherosclerotic plaques.

Acknowledgments

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