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Extracellular Nucleotides Can Induce Chemokine (C-C motif) Ligand 2 Expression in Human Vascular Smooth Muscle Cells

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To understand the roles of purinergic receptors and cellular molecules below the receptors in the vascular inflammatory response, we determined if extracellular nucleotides up-regulated chemokine expression in vascular smooth muscle cells (VSMCs). Human aortic smooth muscle cells (AoSMCs) abundantly express $P2Y_1$, $P2Y_6$, and $P2Y_{11}$ receptors, which all respond to extracellular nucleotides. Exposure of human AoSMCs to NAD^+, an agonist of the human $P2Y_{11}$ receptor, and $NADP^+$ as well as ATP, an agonist for $P2Y_1$ and $P2Y_{11}$ receptors, caused increase in chemokine (C-C motif) ligand 2 gene (*CCL2*) transcript and CCL2 release; however, UPT did not affect CCL2 expression. CCL2 release by NAD^+ and $NADP^+$ was inhibited by a concentration dependent manner by suramin, an antagonist of P2-purinergic receptors. NAD^+ and $NADP^+$ activated protein kinase C and enhanced phosphorylation of mitogen-activated protein kinases and Akt. NAD^+- and $NADP^+$-mediated CCL2 release was significantly attenuated by SP6001250, U0126, LY294002, Akt inhibitor IV, RO318220, GF109203X, and diphenyleneiodium chloride. These results indicate that extracellular nucleotides can promote the proinflammatory VSMC phenotype by up-regulating CCL2 expression, and that multiple cellular elements, including phosphatidylinositol 3-kinase, Akt, protein kinase C, and mitogen-activated protein kinases, are involved in that process.

Key Words: CCL2, NAD^+, P2 receptors, Vascular smooth muscle cell

INTRODUCTION

Chemokines and cytokines play important roles in the initiation and progression of atherosclerosis. They promote recruitment and migration of inflammatory cells into the atherosclerotic lesion and induce activation of endothelial cells and leukocyte subsets, leading to release of inflammatory cytokines and chemokines. These in turn further promote recruitment and activation of leukocytes into the atherosclerotic lesion [1,2]. Among the chemokines, the chemokine (C-C motif) ligand 2 (CCL2), which is also known as monocyte chemoattractant protein (MCP)-1, strongly induces mononuclear cells, predominantly monocytes and lymphocytes, to migrate and infiltrate atherosclerotic lesion through the activation of chemokine receptor 2 (CCR2) [3]. Since CCL2 has strong chemotactic activity toward inflammatory cells, expression of CCL2 or its receptor CCR2 is linked to atherosclerosis. The higher the expression of CCL2 or CCR2, the greater the chance of developing atherosclerosis [4,5]. Conversely, the deletion of CCL2 or the gene for its receptor is followed by a significant reduction in the development of atherosclerotic plaques [6,7]. Therefore, understanding regulation of CCL2 expression is important because of its close association with atherosclerosis.

Extracellular nucleotides function as signaling molecules through P2 purinergic receptors, the plasma membrane receptors for those nucleotides [8]. P2 purinergic receptors are ubiquitously expressed throughout the human body and modulate responses of blood cells (i.e., platelets and granulocytes) and vascular cells (i.e., endothelial cells and smooth muscle cells) [9]. Therefore, P2 receptors regulate physiologic responses such as platelet aggregation, cardiac function, and release of endothelial factors [10]. In addition, P2 receptors are associated with vascular diseases. Activation of P2 receptors with extracellular ATP can induce not only vascular smooth muscle cell (VSMC) proliferation, but also apoptosis of endothelial and immune cells [11-13], processes involved in the evolution of atherosclerotic plaques. However, it is not clear whether P2 receptors are involved in the proinflammatory phenotypic changes of vascular cells.

In the present study, we investigated whether activation of P2 receptors by extracellular nucleotides promoted proinflammatory phenotypic changes in vascular smooth muscle

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ABBREVIATIONS: ATP, adenosine-5'-triphosphate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; UTP, uridine-5'-triphosphate; VSMC, vascular smooth muscle cell.

cells (VSMCs). We found that nucleotides such as NAD^+, $NADP^+$, and ATP enhanced CCL2 expression in human aortic smooth muscle cells (AoSMCs). Moreover, we identified the cellular factors involved in nucleotide-mediated CCL2 expression, and found that mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), Akt, and NADPH oxidase were involved in NAD^+- and $NADP^+$-mediated CCL2 up-regulation.

METHODS

Cell culture and reagents

Human AoSMCs were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained as previously reported [14]. NAD^+, $NADP^+$, RO318220, GF109203X, LY294002, diphenyleneiodonium chloride (DPI), and SP600125 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). U0126, SB202190, Akt inhibitor IV (Akti IV), and anti-phosphorylated Akt antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against phosphorylated ERK and phosphorylated p38 MAPK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and R&D systems (Minneapolis, MN, USA), respectively. Anti phosphorylated JNK antibody was purchased from Stressgen Biotechnologies Corporation (Victoria, BC, Canada).

CCL2 Enzyme-linked immunosorbent assay (ELISA)

The amount of CCL2 released from AoSMCs was determined using a commercially available ELISA kit, according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). AoSMCs were incubated for 12 h in the presence of 1% fetal bovine serum (FBS) and exposed for 9 h to NAD^+, $NADP^+$, or ATP prior to collection of cell culture media. Cell culture media and standards for CCL2 were added to a microtiter plate pre-coated with monoclonal antibody against CCL2. After incubation for 2 h, the plate was washed and incubated with the enzyme-conjugated polyclonal antibody specific for CCL2. The substrate solution was added after several plate washes, and color intensity was measured. The amount of CCL2 present in the medium was determined from a standard curve. Data are expressed as mean±SD.

Reverse transcription (RT) - polymerase chain reaction (PCR)

Total RNAs were extracted from cells and reverse-transcribed for an hour at 42°C with Moloney murine leukemia virus reverse transcriptase. PCR amplification was performed for 30 cycles (94°C for 30 sec; 55°C for 30 sec; 72°C for 30 sec) in the presence of primers. PCR primers for CCL2 were 5'-TGGCTGTGTTTGCTTCTGTC-3' (forward) and 5'-TCTCACTGCCCTATGCCTCT-3' (reverse). PCR primers for GAPDH were 5'-GAGTCAACGGATTTGGTCGT-3' (forward) and 5'-TGTGGTCATGAGTCCTTCCA-3' (reverse). Primers for P2 purinergic receptors were prepared as previously described [14].

PKC activity assay

PKC activity was determined using a PKC activity assay kit (BD Biosciences) according to the manufacturer's instructions. Cells were collected and lysed in ice-cold lysis buffer containing 20 mM 3-morpholinopropanesulfonic acid, 50 mM β-glyceraldehyde phosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA 1% NP40, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethanesulphonyl fluoride, and 10 μg/ml each aprotinin and leupeptin. Cell lysate was collected and centrifuged to obtain supernatant. Purified active PKC provided in the kit and the supernatants were added to wells in a 96-well plate, and PKC reactions were initiated by adding ATP. After incubation for 90 min, the reactions were stopped by aspirating the wells. Phospho-specific substrate antibody was added to each well and incubated for 50 min at room temperature. HRP-conjugated secondary antibody was added and incubated for 30 min at room temperature. After washing, substrate solution was added and incubated at room temperature. Absorbance was measured at 450 nm.

Western blot analysis

Cell lysate was prepared using a lysis buffer [1% SDS, 1 mM $NaVO_3$, 10 mM TrisHCl (pH 7.4)] containing protease inhibitors. The isolated cell lysate was separated by SDS-PAGE and transferred to PVDF membranes. After the membranes were blocked by 1 h incubation in 5% skim milk/0.1% Tween 20 in phosphate buffered saline (PBS), they were incubated overnight at 4°C with appropriate primary antibodies. After three washes using 0.1% Tween20 in PBS, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies. Bands were visualized using a chemiluminescent reagent.

Statistics

Statistical analyses were performed using GraphPad PRISM, version 5.0 (GraphPad Software Inc., San Diego, CA, USA), and $p<0.05$ was considered statistically significant.

RESULTS

Enhancement of CCL2 expression by NAD^+, $NADP^+$, and ATP in human AoSMC

We examined the P2 purinergic receptors found on human AoSMCs. The transcript of the following P2 receptors were readily detected in human AoSMC total RNA that was subjected to RT-PCR: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2X_1$, and $P2X_4$ (Fig. 1A). Among these receptors, $P2Y_6$, $P2Y_{11}$, $P2X_1$, and $P2X_4$ appeared abundantly expressed. We investigated whether NAD^+, an agonist of $P2Y_{11}$, affected expression of chemokines in parallel with $NADP^+$ and ATP, an agonist of $P2Y_2$, $P2Y_{11}$, and several P2X receptors. RT-PCR analysis indicated that both NAD^+ and $NADP^+$ increased CCL2 expression, which was evident by 6 h post-treatment. CCL2 expression was also increased by ATP, but not by UTP, an agonist of $P2Y_2$ and $P2Y_4$ (Fig. 1B). When we examined the effects NAD^+ and $NADP^+$ on expression of the genes for CXCL8 and interleukin (IL)-6, the number of *CXCL8* and *IL6* transcripts were not influenced by treatment using 10^{-4} M NAD^+ and $NADP^+$, concentration which elevated the number of *CCL2* transcripts (Fig. 1C).

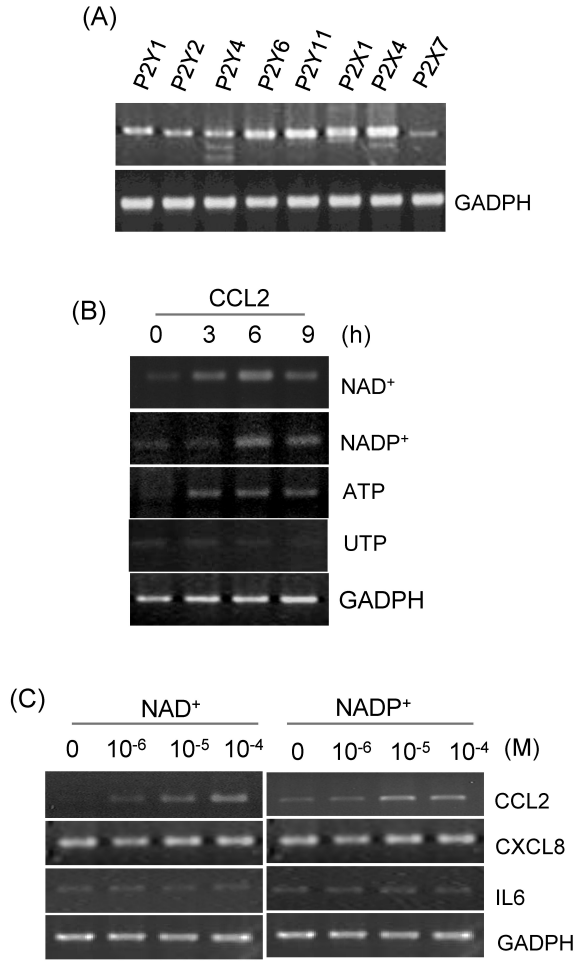

Fig. 1. The effects of extracellular nucleotides on *CCL2* gene transcripts in human VSMCs. (A) Total RNA was isolated from human AoSMCs, and transcripts of the indicated P2 purinergic receptors were identified by RT-PCR. (B) Human AoSMCs were treated with nucleotides for the indicated time periods, and *CCL2* transcripts were amplified by RT-PCR. (C) Human AoSMCs were treated for 6 h with the indicated concentrations of NAD$^+$ or NADP$^+$, and induction of *CCL2* gene transcripts was examined by RT-PCR.

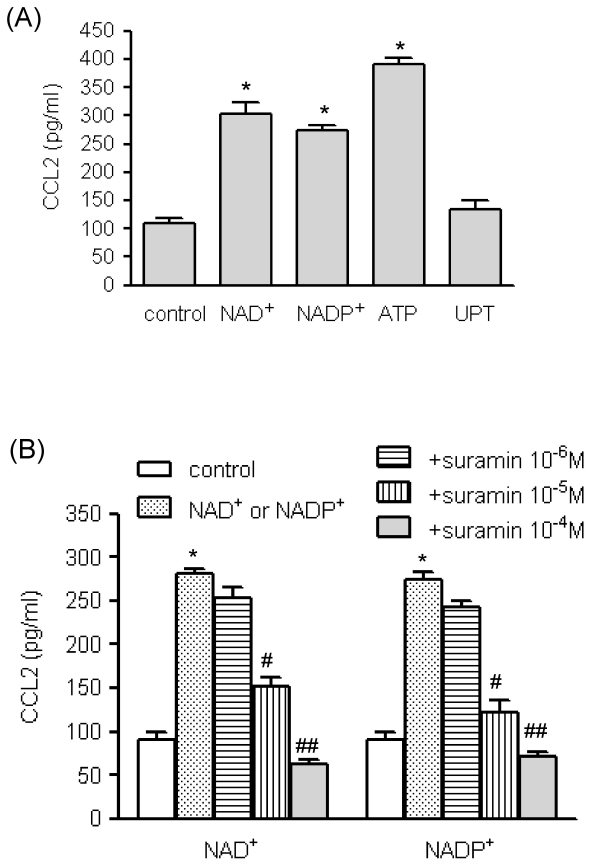

Fig. 2. The effects of nucleotides on NAD$^+$- and NADP$^+$-mediated CCL2 release. (A) Human AoSMCs (1×10^6 cells) were incubated in the absence (control) or presence of indicated nucleotides (10^{-4} M for each nucleotide). Culture medium was collected and the amount of secreted CCL2 was measured by ELISA. *$p<0.001$ vs. control. (B). Human AoSMCs were incubated with NAD$^+$ or NADP$^+$ (10^{-4} M) in the absence or presence of indicated concentrations of suramin. CCL2 released into the medium was measured by ELISA. *$p<0.001$ vs. control; $^{\#}p<0.01$ vs. NAD$^+$ or NADP$^+$, $^{\#\#}p<0.001$ vs. NAD$^+$ or NADP$^+$.

We also determined the effects of the nucleotides on CCL2 protein expression. NAD$^+$, NADP$^+$, and ATP significantly elevated CCL2 release from AoSMCs, but UTP did not (Fig. 2A). Because the cellular and molecular effects of ATP are well documented, we focused on the mechanisms of action of NAD$^+$ and NADP$^+$. We examined whether suramin, an antagonist of purinergic receptors, could modulate the induction of CCL2 expression by NAD$^+$ and NADP$^+$. Suramin inhibited CCL2 secretion from AoSMCs in a concentration-dependent manner, and NAD$^+$- and NADP$^+$-mediated CCL2 release was almost completely blocked in the presence of 10^{-4} M suramin (Fig. 2B).

Involvement of mitogen-activated protein kinases (MAPKs) in NAD$^+$- and NADP$^+$-mediated CCL2 up-regulation

MAPKs play important roles in VSMC secretion of cytokines and chemokines [15,16]. We investigated whether MAPKs affected nucleotide-mediated CCL2 secretion. We found that NAD$^+$ and NADP$^+$ enhanced phosphorylation of MAPKs (Fig. 3A). Phosphorylated extracellular signal-regulated kinase (ERK) and p38 MAPK reached maximum levels 20 min post treatment, and c-jun N-terminal kinase (JNK) was phosphorylated 5 min post treatment with NAD$^+$ or NADP$^+$. To assess the roles of MAPKs on NAD$^+$- and NADP$^+$-mediated CCL2 up-regulation, we applied following MAPK inhibitors; SP600125 (JNK inhibitor), U0126 (ERK inhibitor) and SB202190 (p38 MAPK inhibitor). NAD$^+$-mediated CCL2 release was completely blocked by those inhibitors, and NADP$^+$-induced release of CCL2 protein was inhibited by SP600125 and U0126 (Fig. 3B).

Involvement of Akt, PKC, and NADPH oxidase in NAD$^+$- and NADP$^+$-mediated up-regulation of CCL2

We examined the effects of NAD$^+$ and NADP$^+$ on Akt phosphorylation using Western blot analysis. NAD$^+$ and

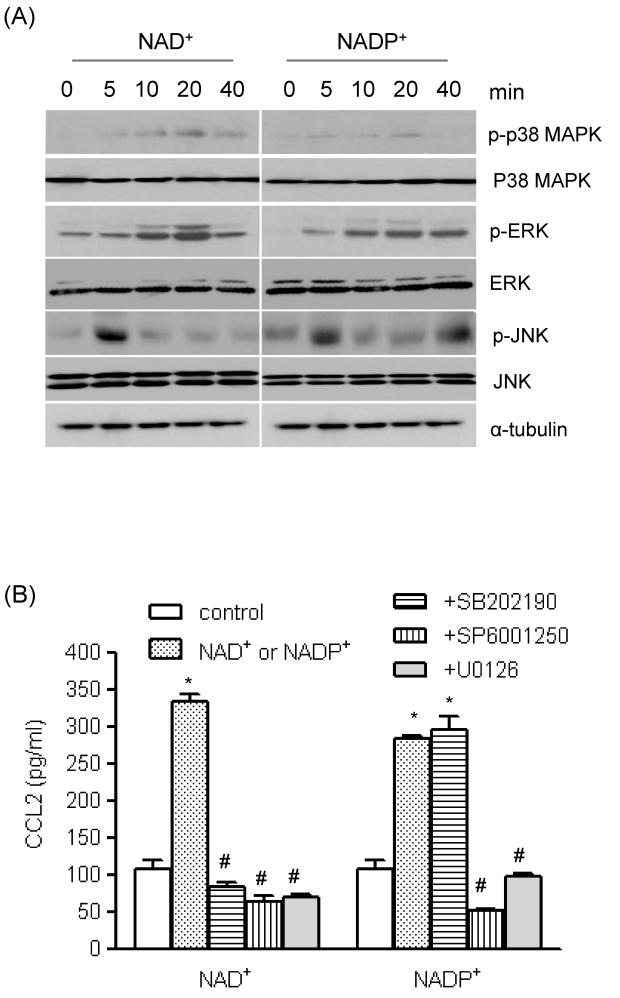

Fig. 3. The roles of MAPKs in NAD^+- and $NADP^+$-mediated CCL2 release. (A) Human AoSMCs were exposed to NAD^+ or $NADP^+$ for the indicated time periods, after which an equal amount of protein was subjected to Western blot analysis using antibodies for α-tubulin and phosphorylated and unphosphorylated forms of ERK, p38 MAPK, and JNK. (B) Human AoSMCs were incubated for an hour with SP600125, U0126, and SB202190 (10 μM each) and stimulated with NAD^+ or $NADP^+$. Culture media were collected to measure the amount of secreted CCL2. *$p<0.001$ vs. control, #$p<0.001$ vs. NAD^+ or $NADP^+$.

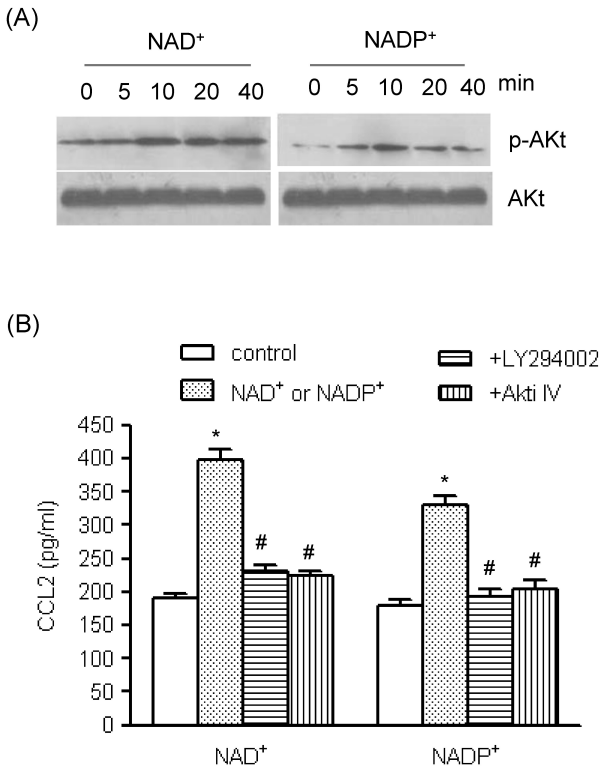

Fig. 4. The roles of Akt pathways in NAD^+- and $NADP^+$-mediated CCL2 release. (A) Human AoSMCs were exposed to NAD^+ or $NADP^+$ for the indicated time periods, after which an equal amount of protein was subjected to Western blot analysis using antibodies for Akt and phosphorylated Akt. (B) Human AoSMCs were incubated for 1 h with LY294002 and Akti IV (10 μM each) and stimulated with NAD^+ or $NADP^+$. The amount of secreted CCL2 was measured by ELISA. *$p<0.001$ vs. control, #$p<0.001$ vs. NAD^+ or $NADP^+$.

$NADP^+$ enhanced Akt phosphorylation, which was maximum 10 min post treatment (Fig. 4A). To investigate whether the Akt pathway was involved in nucleotide-mediated CCL2 expression, we used LY294002, a phosphoinositide 3-kinase (PI3K) inhibitor, and Akti IV. When the effects of LY294002 and Akti IV on CCL2 release were examined by ELISA, both inhibitors profoundly attenuated NAD^+ and $NADP^+$-mediated CCL2 release (Fig. 4B).

AoSMCs express PKC subtypes which play roles in chemokine and cytokine secretion [17]. Examining the effects of NAD^+ and $NADP^+$ on PKC activity, we found that NAD^+ and $NADP^+$ enhanced PKC activity (Fig. 5A). PKC activity was increased 5 min post treatment and remained enhanced up to 30 min post treatment by NAD^+ or $NADP^+$. To assess involvement of PKC in the nucleotide-mediated up-regulation of CCL2, we used two PKC inhibitors, GF109203X and RO318220. Both inhibitors profoundly attenuated NAD^+ and $NADP^+$-mediated CCL2 release (Fig. 5B). We also investigated whether reactive oxygen species (ROS) participated in nucleotide-mediated CCL2 expression, using DPI, an inhibitor of NADPH oxidase. DPI appeared to attenuate NAD^+ and $NADP^+$-mediated *CCL2* expression at the messenger mRNA level and significantly inhibited CCL2 release (Fig. 6).

DISCUSSION

Extracellular nucleotides such as ATP have long-term trophic effects, including smooth muscle cell proliferation via the $P2Y_2$ and/or $P2Y_4$ receptors, which transmit the signals through MAPK pathways [18,19]. In response to the MAPK cascade, two transcription factors, c-fos and c-myc, are synthesized in addition to expression of other immediate early genes prior to downstream gene expression of mitogenesis [20]. In this study, we investigated the short-term actions of extracellular nucleotides, and found that NAD^+ and $NADP^+$, in addition to ATP, promoted a VSMC proinflammatory phenotype by causing elevated CCL2 expression.

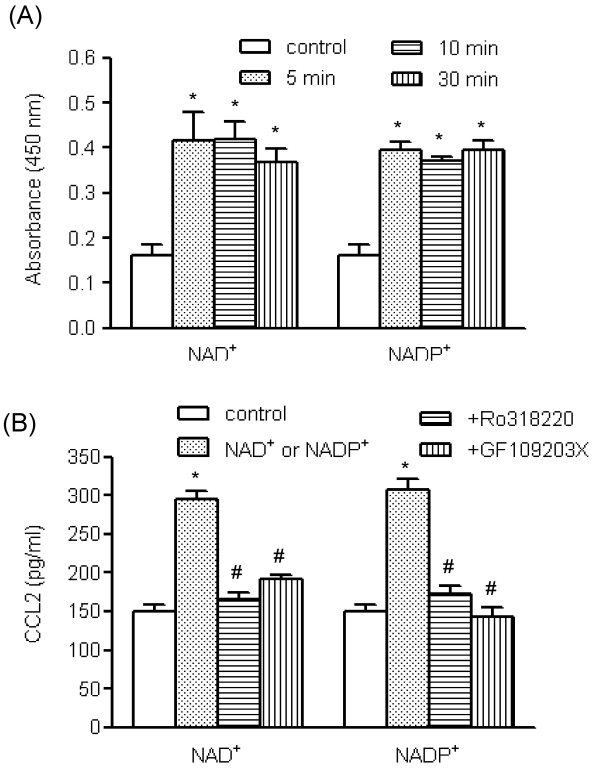

Fig. 5. The roles of PKC in NAD^+- and $NADP^+$-mediated CCL2 release. (A) Human AoSMCs were exposed to NAD^+ or $NADP^+$ for the indicated time periods, after which PKC activity was determined. *$p<0.01$ vs. control. (B) Human AoSMCs were incubated for 1 h with GF109203X (3 μM) and RO318220 (1 μM) and stimulated with NAD^+ or $NADP^+$. The amount of secreted CCL2 was measured by ELISA. *$p<0.001$ vs. control, $^{\#}p<0.001$ vs. NAD^+ or $NADP^+$.

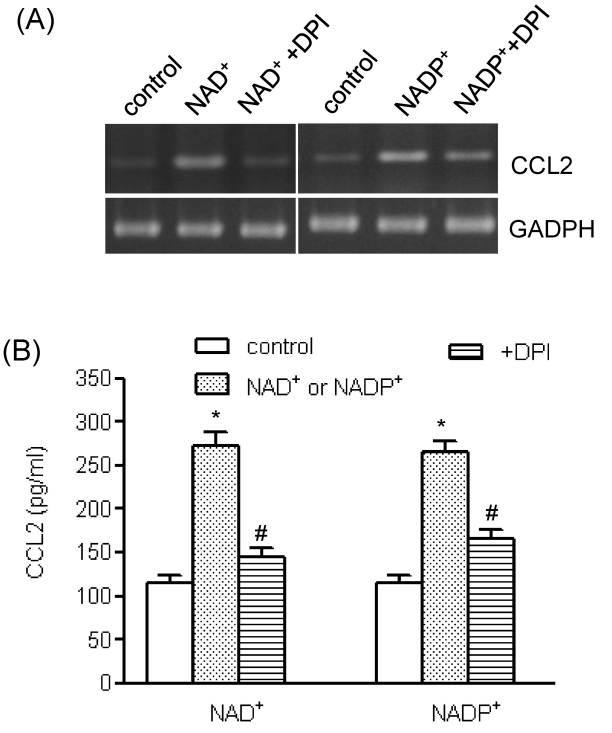

Fig. 6. The roles of NADPH oxidase in NAD^+- and $NADP^+$-mediated CCL2 release. Human AoSMCs were incubated for 1 h with DPI (10 μM) and stimulated with NAD^+ or $NADP^+$. CCL2 gene transcript was amplified by RT-PCR (A), and the amount of released CCL2 was measured by ELISA (B). *$p<0.001$ vs. control, $^{\#}p<0.001$ vs. NAD^+ or $NADP^+$.

NAD^+ and $NADP^+$ enhanced the activity of MAPKs, PKC, and Akt, and pharmacological inhibitors caused attenuated CCL2 expression, which indicates involvement of the signaling pathways in short-term activation of P2 purinergic receptors.

We demonstrated that human AoSMCs abundantly expressed multiple P2 purinergic receptor subtypes, including $P2Y_{11}$ in addition to $P2X_1$, $P2Y_2$ and $P2Y_6$. This finding is consistent with the results of a previous study by Wang et al. Using real-time PCR, they found that P2 receptors were expressed by VSMCs isolated from internal mammary arteries [15]. The effects of $P2Y_2$ and $P2Y_4$ on vascular cells are understood somewhat [18,21]. However, it is not clear what cellular events are mediated and which signaling pathways are activated by $P2Y_6$ and $P2Y_{11}$ receptors on VSMCs. In the present study, we investigated whether $P2Y_{11}$ is involved in the development of a VSMC proinflammatory phenotype of VSMCs using NAD^+, a $P2Y_{11}$ agonist. Stimulation of VSMCs with NAD^+, $NADP^+$, or ATP enhanced CCL2 expression, and CCL2 release from VSMCs by NAD^+ and $NADP^+$ was completely inhibited in a dose-dependent manner by the P2 receptor-specific inhibitor, suramin. These results indicate that P2 purinergic receptors are responsible for NAD^+- and $NADP^+$-mediated CCL2 up-regulation.

We determined which cellular factors were involved in CCL2 expression in response to NAD^+ and $NADP^+$. Since MAPKs are responsible for the mitogenic effects of P2Y receptors [18,22], we investigated whether kinases are also involved in CCL2 expression. NAD^+ and $NADP^+$ treatment of human HAoSMCs led to phosphorylation of p38 MAPK, ERK1/2, and JNK, and NAD^+- and $NADP^+$-mediated CCL2 release was completely blocked by inhibitors of ERK2/1 and JNK. Inhibition of p38 MAPK affected CCL2 release in a different pattern. SB202190 inhibited NAD^+-mediated, but not $NADP^+$-mediated, CCL2 release, which suggests a different role for p38 MAPK in CCL2 release in response to extracellular nucleotides. Together, the results indicated that MAPKs are actively involved in the mechanisms of CCL2 expression by VSMCs responding to extracellular nucleotides.

We have also identified other molecules involved in $P2Y_{11}$ signaling pathways. In a study of the mechanisms involved in ATP-induced proliferation of VSMCs, it was shown that P2Y receptor activation is coupled to a pertussis toxin-insensitive Gq protein, triggering phosphoinositide hydrolysis and subsequent activation of PKC, Raf 1, and MAPK [22,23]. In addition, the mitogenic actions of ATP required PI3K signaling pathways [19]. Therefore, we investigated the roles of PKC and PI3K in CCL2 expression. NAD^+ and $NADP^+$ not only enhanced phosphorylation of Akt but also elevated PKC activity. PI3K, Akt, and PKC inhibitors blocked CCL2 secretion stimulated by NAD^+ and $NADP^+$.

We believe that some PKC subtypes such as PKC-βI, λ, and μ are also involved in CCL2 secretion, because the AoSMCs used in this study have been reported to express those PKC subtypes, but not PKC-α/δ/ξ/γ, βII, ζ, η, and θ subtypes [17].

NADPH oxidases are a major source of ROS, which are involved in atherogenesis, where they act as second messengers that mediate the signaling pathways in VSMC activation and proliferation [24]. We investigated the role of ROS in CCL2 expression, using DPI, an inhibitor of NADPH oxidase inhibitor. DPI attenuated CCL2 expression at both the mRNA and protein levels. However, we were not able to obtain conclusive evidence that NAD^+ and $NADP^+$ significantly enhanced ROS generation in VSMCs exposed to 2,7-dichlorofluorescin diacetate (data not shown).

We showed that P2 purinergic receptor agonists induced expression of *CCL2* gene transcripts and enhanced CCL2 release from VSMCs, and found that MAPKs, PKC, PI3K, Akt, and NADPH oxidase participated in that process. This study, however, did not specifically determined if these factors acted in an independent or cooperative manner to up-regulate CCL2 expression. Further investigation is necessary to elucidate the types of connections or crosstalk that may be occurring in the context of a possible signaling cascade.

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