Moyamoya disease is an unusual form of chronic cerebrovascular occlusive disease that is characterized by progressive stenosis or occlusion at the distal ends of the bilateral internal carotid arteries. The disease peaks or progresses primarily during the first decade of life, and the neurological symptoms depend on the specific arteries that are occluded. The cause of the disease remains unknown. The findings that the incidence of the disease is highest in the bilateral internal carotid arteries suggest the involvement of a genetic factor in its pathogenesis. Previous reports and our findings suggest the involvement of systemic as well as intracranial arterial smooth muscle cells (SMCs) derived from patients with moyamoya disease. Twelve moyamoya and 8 control cell strains were examined. The steady-state levels of prostanoids in the culture medium did not differ between moyamoya and control SMCs. When the cells were stimulated with interleukin-1β (IL-1β), prostaglandin E₂ (PGE₂) release into the medium was significantly greater from moyamoya SMCs than from control SMCs, whereas the amounts of prostacyclin and thromboxane B₂ did not differ. IL-1β–induced PGE₂ production by moyamoya SMCs was completely blocked by the addition of indomethacin or NS-398. IL-1β significantly stimulated cell migration and DNA synthesis in control SMCs but had an inhibitory effect on moyamoya SMCs. The inhibitory effects on the growth and migration of moyamoya SMCs were caused by excessive secretion of PGE₂ and was reversed with indomethacin treatment. Immunofluorescence studies and Western blot analysis showed greater amounts of COX-2 protein expression in IL-1β–stimulated moyamoya SMCs. These findings suggest that moyamoya SMCs respond to inflammatory stimuli to produce excess amounts of PGE₂ through the activation of COX-2, which increases vascular permeability and decreases vascular tone. This facilitates the exposure of vessels to blood constituents and promotes the development of intimal thickening in moyamoya disease.

Key Words: interleukin, moyamoya disease, muscle, smooth, prostanogand
Yamamoto et al. PGE2 Production by Moyamoya SMCs

### TABLE 1. Established Strains of Arterial SMCs Derived From Patients With Moyamoya Disease (HMSMC) and Control Patients (HCSMC)

<table>
<thead>
<tr>
<th>Cell Strain</th>
<th>Onset of Disease</th>
<th>Primary Disease</th>
<th>Donor Artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMSMC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8/M, 7 TIA</td>
<td>Head injury</td>
<td>STA</td>
</tr>
<tr>
<td>5</td>
<td>11/F, 4 TIA</td>
<td>Head injury</td>
<td>STA</td>
</tr>
<tr>
<td>9</td>
<td>9/M, 2 TIA</td>
<td>Craniosynostosis</td>
<td>STA</td>
</tr>
<tr>
<td>16</td>
<td>8/F, 4 TIA</td>
<td>Head injury</td>
<td>STA</td>
</tr>
<tr>
<td>18</td>
<td>8/F, 7 INF</td>
<td>Head injury</td>
<td>STA</td>
</tr>
<tr>
<td>22</td>
<td>13/F, 13 ICH</td>
<td>Head injury</td>
<td>STA</td>
</tr>
<tr>
<td>23</td>
<td>2/M, 2 TIA</td>
<td>Cranial bone tumor</td>
<td>STA</td>
</tr>
<tr>
<td>30</td>
<td>9/M, 9 TIA</td>
<td>Head injury</td>
<td>STA</td>
</tr>
<tr>
<td>32</td>
<td>7/M, 2 TIA</td>
<td>Head injury</td>
<td>STA</td>
</tr>
<tr>
<td>36</td>
<td>16/F, 8 TIA</td>
<td>Head injury</td>
<td>STA</td>
</tr>
<tr>
<td>38</td>
<td>9/F, 9 ICH</td>
<td>Head injury</td>
<td>STA</td>
</tr>
<tr>
<td>50</td>
<td>15/M, 6 INF</td>
<td>Head injury</td>
<td>STA</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>9.6 (3.8)*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| HCSMC       |                  |                 |              |
| 1           | 1/F, 18/Head injury | Head injury | STA         |
| 4           | 18/F, 10/Head injury| Head injury | STA         |
| 5           | 10/M, 6/Head injury | Craniosynostosis| STA        |
| 7           | 6/F, 14/Head injury | Cranial bone tumor| STA  |
| 14          | 1/M, 16/Head injury | Head injury | STA         |
| 16          | 17/M, 17/Head injury| Head injury | STA         |
| 17          | 2/F, 18/Head injury | Cranial bone tumor| STA       |
| 18          | 15/F, 15/Head injury| ICH           | STA         |
| Mean (SD)   | 8.8 (7.2)        |                 |              |

ICH indicates intracerebral hemorrhage; INF, infarction; TIA, transient ischemic attack; STA, superficial temporal artery.
*NS vs controls (HCSMC).

ease.26,27 We recently found that moyamoya SMCs show distinct migratory and proliferative responses through an NO-independent pathway when stimulated by IL-1.28 In the present study, we examined prostanoid production and COX-2 expression in IL-1β–stimulated SMCs derived from patients with moyamoya disease and compared the results with those in SMCs from age-matched control subjects. We show that the distinct responses of moyamoya SMCs to inflammatory stimuli may contribute to the pathological process in the vascular wall of patients with moyamoya disease.

### Materials and Methods

#### Cell Culture

Arterial SMC strains from patients with moyamoya disease (HMSMC) and from control subjects (HCSMC) were established as described previously.26 We used 12 SMC strains from patients with moyamoya disease and 8 strains from control subjects (Table 1). The cells were cultured in 5 mL of Eagle’s MEM (GIBCO) supplemented with 15% FBS (Biocell) at 37°C under humidified 5% CO2/95% air and subcultured at a 1:2 split ratio. For the present study, we used cells at 8 to 14 passages.29

#### Determination of Prostanoid Production

Medium was replaced with fresh medium containing 0.5% FBS and 500 U/mL IL-1β (Otsuka Pharmaceutical) with or without 1 μg/mL indomethacin (Sigma Chemical Co), a nonselective COX inhibitor; 1 μM/L NS-398 (BIOMOL Research Laboratories), a COX–2–selective inhibitor; or 5 μM/L arachidonic acid (AA; Sigma Chemical Co), and the cells were incubated for 48 hours at 37°C. PGE2, PGI2, and thromboxane (TX)B2 secreted into the medium were measured with the use of enzyme immunoassay kits for PGE2, 6-keto-PGF1α, and TXB2 (Cayman Chemical), respectively.

#### Migration Assay

SMC migration was monitored in a microchemotaxis chamber (Neuro Probe) with the use of polycarbonate membranes with 8-μm pores, as described previously.28 Cell suspension was placed in the lower compartment with or without indomethacin (1 μg/mL). The lower compartment contained MEM containing 2% FBS and test reagents (500 U/mL IL-1β and 0.4 to 200 nmol/L PGE2, Paesel). The samples were incubated in a CO2 incubator for 18 hours at 37°C.

#### Incorporation of 5-Bromo-2′-Deoxyuridine Into Cellular DNA

5-Bromo-2′-deoxyuridine (BrdU) incorporation was measured with an immunoperoxidase technique (Amersham Corp), as previously described.31 Quiescent SMCs were incubated in MEM containing 0.5% FBS, test reagents, and a labeling regent (BrdU) for 48 hours. The test reagents were IL-1β (500 U/mL), indomethacin (1 μg/mL), and PGE2 (0.4 to 200 nmol/L).

#### Immunocytochemistry

Quiescent SMCs were incubated with IL-1β (500 U/mL) for 9 hours and fixed in acetone/methanol (1:1) at 4°C for 30 minutes. The cells were preincubated with a 1:250 dilution of normal rabbit serum at 4°C for 30 minutes, with a 1:250 dilution of goat anti–COX-1 or anti–COX-2 antibody (Santa Cruz Biotechnology) at 4°C for 1 hour, and then with a 1:250 dilution of rhodamine-conjugated rabbit anti-goat IgG (ICN Biomedicals) at 24°C for 45 minutes.

#### Western Blot Analysis

Western blot analysis was performed with 10% acrylamide gels, as described previously.32 After blocking, membranes were incubated with a 1:250 dilution of normal rabbit serum at 4°C for 1 hour, and then with a 1:250 dilution of rhodamine-conjugated rabbit anti-goat IgG (ICN Biomedicals) at 24°C for 45 minutes.

#### Statistical Analysis

Values are given as mean±SD. Differences in data between groups were assessed with the use of an unpaired t test. A value of P<0.05 was considered statistically significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

### Results

#### Prostanoid Production in Culture Medium of Arterial SMCs Derived From Patients With Moyamoya Disease

The steady-state levels of PGE2, PGI2, and TXB2 production in HMSMC strains were low and not significantly different from those in HCSMC strains (Figure 1). Although IL-1β promoted both PGE2 and PGI2 production in the culture medium of HCSMC and HMSMC strains, the levels of IL-1β–induced PGE2 production in HMSMC (44.8-fold) were significantly greater than those in HCSMC (10.2-fold).
by the addition of AA, but the amount did not differ significantly from that produced by control strains (Figure 2). The AA-stimulated PGE₂ production in HMSCM strains was significantly increased by IL-1β compared with that in HCSMC strains.

**Cell Migration and DNA Synthesis**

The number of migrating cells in serum-deficient medium without test mitogens (controls) did not differ between HCSMC (80.4±17.2) and HMSCM (81.1±11.1) strains. IL-1β stimulated cell migration in HMSCM strains, although it significantly inhibited migration in HCSMC strains (Table 2). IL-1β also stimulated BrdU incorporation into cellular DNA in control SMCs but had a rather inhibitory effect on DNA synthesis in moyamoya SMCs (Table 2). The basal labeling indices (controls) were 17.0±4.8% for HCSMC strains and 18.4±5.0% for HMSCM strains (P=NS). When prostaglandin synthesis was inhibited with indomethacin, IL-1β significantly stimulated cell migration and BrdU incorporation in both HMSCM and HCSMC strains (Table 2). High concentrations (20 to 200 nmol/L) of exogenous PGE₂ induced the inhibition of IL-1β-stimulated cell migration and DNA synthesis in HCSMC and HMSCM strains, but lower concentrations (0.4 to 4 mmol/L) had no detectable inhibitory effects (Table 2). The higher PGE₂ concentrations correspond to those produced by IL-1β-stimulated HMSCM, and the lower concentrations correspond to those produced by IL-1β–stimulated HCSMC (Figure 1).

**COX-2 Expression**

We examined the expression of COX-1 and COX-2 proteins in cultured arterial SMCs through immunofluorescence study. No positive immunostaining was found in both moyamoya and control SMCs in the absence of the primary antibody or in the nonimmune goat IgG (Figure 3, A and B). COX-2 protein was hardly detected in moyamoya and control SMCs grown to confluence (Figure 3, C and D). IL-1β stimulated the expression of COX-2 protein in both moyamoya and control SMCs (Figure 3, E and F); however, the increase in the number of COX-2–positive cells was significantly (P<0.001) greater in moyamoya SMCs (16.5±2.6%) than in control SMCs (5.3±2.1%). In contrast, COX-1 immunoreactivity did not differ between moyamoya (6.5±3.2%) and control (4.8±2.3%) SMCs in the presence of IL-1β and between moyamoya (5.9±3.7%) and control (5.0±1.8%) SMCs in the absence of IL-1β.

Immunoblot analysis showed that the expression of COX-2 protein was hardly detected in homogenates of moyamoya and control SMCs in the absence of IL-1β (Figure 4). The immunoreactive band to COX-2 increased clearly in both moyamoya and control SMCs in the presence of IL-1β, although the expression of COX-1 protein was not stimulated by IL-1β (Figure 4). The relative density of the COX-2 protein in moyamoya cell strains was significantly (P<0.001) higher than that in control cell strains (Figure 4).

**Discussion**

Previous studies have suggested that moyamoya disease is an acquired disorder in which both an immunologic vascular...
reaction and subsequent inflammation play important roles. Recent evidence indicates that inflammatory stimuli and the subsequent response of inflammatory cells may produce a proliferative response in SMCs in the thickened intima of patients with moyamoya disease. In the present study, we examined the production of prostanoids, which act as mediators that modulate vascular functions when stimulated by proinflammatory cytokines such as IL-1, in arterial SMCs derived from patients with moyamoya disease. IL-1 stimulates PGE$_2$ and PGI$_2$ production in both moyamoya and control SMCs. IL-1 is a multipotent inflammatory mediator that may play a central role in vascular pathophysiology. Previous reports indicate that IL-1 stimulates the migration and proliferation of aortic SMCs, whereas others have shown it to produce do not differ between moyamoya and control SMCs. However, the level of IL-1–stimulated PGE$_2$ production by moyamoya SMCs is significantly greater than that by control SMCs. NO stimulated by IL-1 also acts as a mediator that modulates vascular functions, but IL-1–induced NO production is found to be almost the same in HMSMC and HCMSC strains.

### Table 2. Effects of Exogenous PGE$_2$ on Migration of and BrdU Incorporation Into HMSMC and HCSMC Strains

<table>
<thead>
<tr>
<th></th>
<th>Migration</th>
<th></th>
<th>BrdU Incorporation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HMSMC</td>
<td>HCSMC</td>
<td>HMSMC</td>
<td>HCSMC</td>
</tr>
<tr>
<td>Control</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>IL-1$\beta$</td>
<td>1.42±0.21*</td>
<td>0.55±0.10†</td>
<td>$ 2.28±0.59*    $</td>
<td>0.86±0.30§</td>
</tr>
<tr>
<td>IL-1$\beta$+INDO</td>
<td>1.59±0.21†</td>
<td>1.26±0.19‡</td>
<td>$ 3.28±0.66†    $</td>
<td>2.00±0.36‡</td>
</tr>
<tr>
<td>+PGE$_2$ (0.4 nmol/L)</td>
<td>1.54±0.14†</td>
<td>1.20±0.07‡</td>
<td>$ 3.33±0.41†    $</td>
<td>2.11±0.45‡</td>
</tr>
<tr>
<td>+PGE$_2$ (4.0 nmol/L)</td>
<td>1.50±0.11†</td>
<td>1.20±0.10‡</td>
<td>$ 3.12±0.21†    $</td>
<td>1.98±0.55‡</td>
</tr>
<tr>
<td>+PGE$_2$ (20 nmol/L)</td>
<td>0.56±0.10†</td>
<td>0.53±0.09‡</td>
<td>$ 0.56±0.11†    $</td>
<td>0.65±0.21†</td>
</tr>
<tr>
<td>+PGE$_2$ (200 nmol/L)</td>
<td>0.58±0.17†</td>
<td>0.54±0.11‡</td>
<td>$ 0.62±0.17†    $</td>
<td>0.50±0.11†</td>
</tr>
</tbody>
</table>

INDO indicates indomethacin. Results are given as multiples of the number of migrating cells and multiples of the labeling indices of cultures without test reagents (controls).

$^*$P<0.01, †P<0.001 compared with cells treated without test reagents (controls).

$^\dagger$P<0.01, $^\ddagger$P<0.001 compared with control cells (HCSMC).

Figure 3. Immunostaining for COX-2 protein on moyamoya (B, D, and F) and control (A, C, and E) SMCs. Cells grown on Lab-Tek chamber glass slides were incubated with fresh medium with (E and F) or without (C and D) IL-1$\beta$ (500 U/mL) for 9 hours. The cells fixed in acetone/methanol were incubated with (C through F) or without (A and B) an anti–COX-2 antibody and then with rhodamine-conjugated anti-goat IgG. Confocal micrograms were taken with a Bio-Rad MRC 1000 laser scanning confocal imaging system connected to a Zeiss Axiohot. Positive immunostaining for COX-2 protein is observed in cell cytoplasm. Bar, 50 μm.
The synthesis of PGs that have diverse biological effects on vascular SMCs. IL-1 reportedly stimulates cells to produce platelet-derived growth factor-AA, a positively acting mitogen, and stimulates cells to produce PG E2 and NO, both of which have inhibitory effects on cell migration and DNA synthesis in moyamoya SMCs. As we reported recently, IL-1β was found to significantly stimulate cell migration and DNA synthesis in moyamoya SMCs. The inhibitory effect of IL-1β on cell migration and DNA synthesis in moyamoya SMCs corresponds to the elevated levels of PGE2, but not NO, production. Indomethacin treatment suppresses PG synthesis and results in stimulatory effects by IL-1β on cell migration and DNA synthesis in moyamoya SMCs. Furthermore, the higher concentrations of exogenous AA into PGE2 by moyamoya SMCs in the presence of IL-1β is significantly greater than that by control SMCs. The conversion of exogenous AA into PGE2 by moyamoya SMCs in the presence of IL-1β is significantly greater than that by control SMCs but does not differ between SMCs in the absence of IL-1β. IL-1β-induced prostanooid production by both HMSCMs and HCSMCs is completely blocked by the addition of NS-398 (1 μmol/L), a COX-2-selective inhibitor.

Our findings strongly suggest that inflammatory stimuli and the subsequent inflammatory cell response stimulate the overproduction of PGE2 through an IL-1β-induced COX-2 pathway in SMCs in moyamoya disease.

PGs of the E series are thought to modulate vasodilation and vascular permeability. The excessive amounts of PGE2 released from moyamoya arterial SMCs through COX-2 activation by inflammatory stimuli may increase vascular permeability and decrease vascular tone, facilitating exposure of the vessels to blood constituents, including growth factors and cytokines that might induce and promote the development of intimal thickening in moyamoya disease. The excessive amounts of PGE2 also inhibit the migration and proliferation of SMCs due to excessive autocrine PGE2 production from moyamoya SMCs.

The synthesis of PGs that have diverse biological effects on the vasculature is regulated by two successive metabolic steps: the release of AA from membranous phospholipids and its conversion to PGs. Two COX isoforms, COX-1 and COX-2, are the key enzymes that convert AA to PGs. COX-1 is constitutively expressed in most tissues. In healthy vessels, PGI2, which is a protective, “antiatherogenic” mediator, is formed predominantly in the endothelial layer via the actions of constitutive COX-1. In contrast, COX-2 is undetectable under physiological conditions but is markedly induced by several cytokines and growth factors in vascular cells. COX-2 limits the proliferation of human vascular SMCs. COX-2 is thought to be involved in the overproduction of prostanooids under pathological conditions such as acute and chronic inflammatory disorders and appears to be expressed only by specific stimulatory events. Studies in animals show that vessels damaged by angioplasty or pinch express COX-2, an event that may account for an increased release of protective PGI2. Bishop-Bailey et al show that IL-1 induces COX-2 in human vessels, with a pattern of prostanooid release of PGE2>PGI2>TXB2. The formation of PGE2 is mediated primarily by IL-1β-induced COX-2 in SMCs and macrophages. In the present study, protective PGI2 is released predominantly in IL-1β-stimulated control SMCs. The release of PGI2 may represent an endogenous defense mechanism against endothelial damage.

In contrast, the expression of COX-2 protein and subsequent release of PGE2 are significantly up-regulated in IL-1β-stimulated moyamoya SMCs compared with control SMCs. The conversion of exogenous AA into PGE2 by moyamoya SMCs in the presence of IL-1β is significantly greater than that by control SMCs but does not differ between SMCs in the absence of IL-1β. IL-1β-induced prostanooid production by both HMSCMs and HCSMCs is completely blocked by the addition of NS-398 (1 μmol/L), a COX-2-selective inhibitor. The synthesis of PGE2 has diverse biological effects on the vasculature is regulated by two successive metabolic steps: the release of AA from membranous phospholipids and its conversion to PGs. Two COX isoforms, COX-1 and COX-2, are the key enzymes that convert AA to PGs. COX-1 is constitutively expressed in most tissues. In healthy vessels, PGI2, which is a protective, “antiatherogenic” mediator, is formed predominantly in the endothelial layer via the actions of constitutive COX-1. In contrast, COX-2 is undetectable under physiological conditions but is markedly induced by several cytokines and growth factors in vascular cells. COX-2 limits the proliferation of human vascular SMCs. COX-2 is thought to be involved in the overproduction of prostanooids under pathological conditions such as acute and chronic inflammatory disorders and appears to be expressed only by specific stimulatory events. Studies in animals show that vessels damaged by angioplasty or pinch express COX-2, an event that may account for an increased release of protective PGI2. Bishop-Bailey et al show that IL-1 induces COX-2 in human vessels, with a pattern of prostanooid release of PGE2>PGI2>TXB2. The formation of PGE2 is mediated primarily by IL-1β-induced COX-2 in SMCs and macrophages. In the present study, protective PGI2 is released predominantly in IL-1β-stimulated control SMCs. The release of PGI2 may represent an endogenous defense mechanism against endothelial damage.

In contrast, the expression of COX-2 protein and subsequent release of PGE2 are significantly up-regulated in IL-1β-stimulated moyamoya SMCs compared with control SMCs. The conversion of exogenous AA into PGE2 by moyamoya SMCs in the presence of IL-1β is significantly greater than that by control SMCs but does not differ between SMCs in the absence of IL-1β. IL-1β-induced prostanooid production by both HMSCMs and HCSMCs is completely blocked by the addition of NS-398 (1 μmol/L), a COX-2-selective inhibitor. Our findings strongly suggest that inflammatory stimuli and the subsequent inflammatory cell response stimulate the overproduction of PGE2 through an IL-1β-induced COX-2 pathway in SMCs in moyamoya disease.

PGs of the E series are thought to modulate vasodilation and vascular permeability. The excessive amounts of PGE2 released from moyamoya arterial SMCs through COX-2 activation by inflammatory stimuli may increase vascular permeability and decrease vascular tone, facilitating exposure of the vessels to blood constituents, including growth factors and cytokines that might induce and promote the development of intimal thickening in moyamoya disease. The excessive amounts of PGE2 also inhibit the migration and proliferation of SMCs that might be necessary for the rapid repair of vascular wall injury, resulting in the continued increase in vascular permeability and facilitating the prolonged exposure of the vessels to blood constituents. The continued increase in vascular permeability may be much more important in neointimal accumulation than the exposure to excess individual growth factors. In addition, PGE2 and IL-1 are potent stimulators of angiogenesis and they induce the expression of vascular endothelial cell growth factor, which stimulates both angiogenesis and vascular permeability. The induction of vascular endothelial cell growth factor by PGE2 and IL-1 may be an important mechanism in inflammatory angiogenesis.
may directly or indirectly play an important role in angiogenesis in moyamoya disease.

The mechanism of the specific response of moyamoya SMCs to IL-1β is presently unknown. The distinct increase in PGE₂, but not NO, production in moyamoya SMCs may indicate altered intracellular signaling pathways by which IL-1 induces NO and PG synthesis. Recent findings demonstrate that COX-2–selective inhibitors have excellent anti-inflammatory properties. Our findings suggest a possible interaction between the immune system and the vessel wall in moyamoya disease and serve as a basis for the clinical use of nonsteroidal anti-inflammatory drugs in the treatment of moyamoya disease.

Acknowledgments
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References


Increase in Prostaglandin E₂ Production by Interleukin-1β in Arterial Smooth Muscle Cells Derived From Patients With Moyamoya Disease
Mari Yamamoto, Masaru Aoyagi, Naomi Fukai, Yoshiharu Matsushima and Kiyotaka Yamamoto

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Materials and Methods

Cell Culture
Arterial SMC strains from patients with moyamoya disease (HMSMC) and control subjects (HCSMC) were established as described previously. Arterial specimens were obtained from branches of scrap arteries (superficial temporal arteries) requiring division during indirect bypass or other cranial surgical procedures. We used twelve SMC strains from moyamoya patients and eight from control subjects (Table 1). The age of the moyamoya patients was 9.6 ± 3.8
(mean ± SD) years and the age at disease onset was 6.1 ± 3.4 Years. No associated diseases were found in any of the 12 patients with moyamoya disease. The age of control subjects was 8.8 ± 7.2 years, which was not statistically different from that of the moyamoya patients. Informed consent was obtained from the patients or their relatives and the study was approved by the Ethical Committee of the Tokyo Metropolitan Institute of Gerontology.

The cells were cultured in 60-mm Falcon dishes in 5 mL of Eagle’s minimum essential medium (MEM, GIBCO) supplemented with 15% fetal bovine serum (FBS, Biocell, 62018304) at 37°C under humidified 5% CO2-95% air. Confluent cultures were treated with 0.25% trypsin-0.02% EDTA for 10 minutes at 37°C and subcultured at a 1:2 split ratio. The number of cells was counted with a hemocytometer after trypsin treatment. For the present study, we used cells at 8-14 passages (within 50% of the final population doubling levels) that showed no signs of senescence in vitro. The cells were carefully examined for mycoplasma contamination by the method described previously.

**Determination of Prostanoid Production**

SMCs grown to confluence were washed with MEM containing 0.5% FBS. The medium was replaced with fresh medium containing 0.5% FBS and 500 units/mL (1.5 nmol/L) IL-18 (Otsuka Pharmaceutical Co) with or without 1 μg/mL indomethacin (Sigma Chemical Co), a non-selective COX inhibitor, or 1 μmol/L NS-398 (Biomol), a COX-2 selective inhibitor, and the cells were incubated for 48 hours at 37°C. In some experiments, the cells were incubated in the medium with 5 μmol/L arachidonic acid (AA, Sigma). The medium was collected and filtered through a 0.22-μm filter. PGE2, PGI2, and TXB2
secreted into the medium were measured with enzyme immunoassay kits for \textit{PGE}_2, 6-keto-prostaglandin \textit{Fla}, and \textit{TXB}_2 (Cayman Chemical Co), respectively.

\textbf{Migration Assay}

SMC migration was monitored in a Micro Chemotaxis Assembly (Neuro Probe) using polycarbonate membranes with 8-\textmu m pores, as described previously.\cite{28} Briefly, SMCs grown to confluence were suspended in MEM containing 2\% FBS, and 220 \mu L of cell suspension (1 \times 10^5 \text{ cells/mL}) was placed in the upper compartment of the chamber with or without indomethacin (1 \mu g/mL). The lower compartment contained 30 \mu L MEM containing 2\% FBS and test reagents. The samples were incubated in a CO2 incubator for 18 hours at 37°C. Nonmigrating cells on the upper surface were scraped off gently, and the membranes were fixed in methanol for 30 minutes at room temperature and stained with Diff-Quick solution. SMCs migrating to the lower surface of the membranes were quantified in five or more randomly selected high fields (x400). The area was measured with an image analyzer (SPICCA-II, Olympus). The test reagents were \textit{IL-1\beta} (500 units/ml) and \textit{PGE}_2 (0.4-200 nmol/L, Paesel GmbH). The assays were performed in a blinded fashion.

\textbf{Incorporation of 5-Bromo-2'-deoxyuridine (BrdU) into Cellular DNA}

BrdU incorporation into cellular DNA was measured using an immunoperoxidase technique (cell proliferation kit; Amersham), as previously described.\cite{31} Briefly, SMCs grown to confluence were arrested in MEM containing 0.5\% FBS for 24 hours and then incubated in MEM containing 0.5\% FBS, test reagents, and a labeling regent
(BrdU) for 48 hours. The percentage of labeled nuclei was calculated by counting more than 200 cells in each experiment. The test reagents were IL-18 (500 units/ml), indomethacin (1 μg/mL), and PGE₂ (0.4-200 nmol/L). The experiments were performed in a blinded fashion.

**Immunocytochemistry**

SMCs grown to confluence on Lab-tek chamber glass slides (Nunc) were arrested in MEM containing 0.5% FBS at 37°C for 24 hours and then incubated with IL-18 (500 units/mL) for 9 hours. The cells were fixed in acetone:methanol (1:1) at 4°C for 30 minutes and preincubated with a 1:250 dilution of normal rabbit serum at 24°C for 30 minutes. The cells were incubated with a 1:250 dilution of goat anti-COX-1 or anti-COX-2 antibody (Santa Cruz Biotechnology, Inc) at 24°C for 1 hour and then with a 1:250 dilution of rhodamine-conjugated rabbit anti-goat IgG (ICN Biomedicals, Inc) at 24°C for 45 minutes. As negative controls, cells were processed with pre-immune goat serum or without primary antibody. Confocal micrographs were taken with a Bio-Rad MRC 1000 laser scanning confocal imaging system connected to a Zeiss Axiophot.

**Western blot analysis**

SMCs grown to confluence were arrested in MEM containing 0.5% FBS at 37°C for 24 hours and then incubated with IL-18 (500 units/mL) for 9 hours. Quiescent cells were solubilized in lysis buffer. Equal amounts of protein were loaded on 10% acrylamide slab gels and developed at 25 mA for 2 hours. Samples were transferred from the slab gel to Clearblot-P membranes (ATT0 Co) at 1.5 mA/cm² for 2 hours. After blocking with 0.1% casein and 0.1% gelatin for 1 hour,
the membranes were incubated with a 1:250 dilution of goat anti-COX-1 or anti-COX-2 antibody at 24°C for 1 hour and then with a 1:500 dilution of peroxidase-conjugated rabbit anti-goat IgG (ICN Biomedicals, Inc) at 24°C for 45 minutes. Color was developed using 4CN plus (DuPont NEN Research Products). Densitometric scanning was performed to compare relative protein levels using NIH Images for the Macintosh.

**Statistical analysis**

Data are expressed as means ± SD. Differences in data between groups were assessed by unpaired *t* test. A value of *P*<0.05 is considered statistically significant.