Aspirin Attenuates Cytomegalovirus Infectivity and Gene Expression Mediated by Cyclooxygenase-2 in Coronary Artery Smooth Muscle Cells

Edith Speir, Zu-Xi Yu, Victor J. Ferrans, Eng-Shang Huang, Stephen E. Epstein

Abstract—Human cytomegalovirus (CMV) infection of smooth muscle cells generates reactive oxygen species (ROS) and thereby activates nuclear factor κB (NFκB), which causes expression of viral and cellular genes involved in immune and inflammatory responses. These changes could account for the mounting evidence suggesting that CMV may contribute causally to restenosis and atherosclerosis. We found that CMV induces ROS, at least partly, through a cyclooxygenase-2 (COX-2)–dependent pathway. Moreover, the viral immediate-early (IE) gene products, IE72 and IE84, have the capacity to transactivate the COX-2 promoter. Aspirin and indomethacin, both cyclooxygenase inhibitors as well as direct ROS scavengers, reduce CMV-induced ROS, probably through both of these activities. Sodium salicylate also has antiviral effects as the result of its potent antioxidant properties. Furthermore, by reducing ROS, aspirin and sodium salicylate inhibit CMV-induced NFκB activation, the ability of IE72 to transactivate its promoter, CMV IE gene expression after infection of SMCs, and CMV replication in SMCs. This is the first time aspirin has been shown to have antiviral effects. Thus, it is possible that aspirin has previously unrecognized therapeutic effects in various clinical situations, such as in viral infections (when used as an antipyretic agent) and in atherosclerosis (when used as an antiplatelet agent). (Circ Res. 1998;83:210-216.)

Key Words: antioxidant • atherosclerosis • cyclooxygenase • herpesvirus • salicylate

After primary infection, cytomegalovirus (CMV), like other herpesviruses, establishes lifelong persistence in the host. Although CMV is thought to cause clinically important infection only in newborns and in immunocompromised patients, accumulating evidence suggests that this virus may play a role in the development of restenosis after coronary angioplasty1–3 and in the genesis of atherosclerosis.4

We recently demonstrated that CMV infection of smooth muscle cells (SMCs) generates intracellular reactive oxygen species (ROS) within minutes of infection and that the resulting ROS contribute to nuclear factor κB (NFκB) activation.5 NFκB stimulates the expression of many cellular genes and their products, including cytokines and adhesion molecules, which are involved in immune and inflammatory responses.6 Because the CMV major immediate-early promoter (MIEP) has 4 NFκB-binding sites, activation of NFκB is also critical for MIEP activation and eventual expression of all viral gene products, including the immediate-early (IE) major gene product IE72. In turn, IE72 transactivates its own promoter through the multiple NFκB sites.7 Furthermore, angioplasty-induced injury to the vessel wall and reperfusion after balloon angioplasty produce ROS8 and cytokines. The resulting activation of NFκB can in turn stimulate the MIEP present in latently infected cells and thereby contribute to reactivation of latent CMV.

Because of the critical role in both viral and cellular gene expression, CMV-induced ROS generation might constitute an excellent target for any therapeutic attempt to inhibit those cellular changes that are mediated by CMV infection and that might contribute to either restenosis or atherosclerosis. Indeed, we previously demonstrated that after CMV infection of SMCs, antioxidants inhibit CMV IE gene expression and viral replication.5 Further insights to refine such a strategy require identification of the cellular pathways responsible for generating ROS after CMV infection.

Recent studies have shown that CMV infection of human cells9–11 leads to stimulation of arachidonic acid (AA) release. Although the components downstream of AA release that are responsible for the CMV-stimulated ROS generation in SMCs have not been defined, one such component may involve cyclooxygenase (COX), a major enzyme system by which AA metabolism leads to the generation of ROS. If so, this may have therapeutic implications. Thus, aspirin is commonly used in patients with atherosclerosis. If COX is involved in CMV-induced generation of ROS and if CMV plays a causal role in restenosis and in atherosclerosis, then aspirin, a potent inhibitor of COX, might exert therapeutic effects in such patients through its antiviral properties, in addition to its antiplatelet actions.
Two isoforms of COX, encoded by different genes, catalyze the reactions whereby eicosanoids and ROS are formed from AA. COX-1 is constitutively expressed and appears to mediate housekeeping functions. COX-2 is an inducible enzyme that is induced by various stimuli, many of which appear to exert their effects through the generation of ROS. Furthermore, the COX-2 promoter contains 2 NFκB sites, which are important for cytokine-induced COX-2 transcription. In this investigation we determined whether COX-2 mediates the CMV-induced generation of ROS and whether inhibition of the generation of ROS might serve as a potential target to inhibit CMV gene expression and replication in SMCs.

Materials and Methods

Cells, Viruses, Plasmids, Antibodies, and Drugs

Human coronary artery SMCs at passages 4 to 6 were grown as described previously in their optimum medium (Clonetics). Human kidney 293 cells were purchased from American Type Culture Collection (ATCC) and grown according to ATCC instructions. Human CMV, Towne strain, was passaged in human fibroblasts (HEL299, ATCC) and titrated as described before. Because the virus was harvested in the supernatant of infected fibroblasts in cell culture, we obtained virus-free supernatant by ultracentrifugation for use as negative controls in all experiments. The experiments were repeated with virus purified by ultracentrifugation as follows: Supernatant media of CMV-infected HEL299 cells (containing virus released by the cells) was centrifuged at 3000 rpm to remove cell debris. The supernatant (28 mL) was then carefully layered on a 0.5 mol/L sucrose sterilized in MEM cushion and centrifuged in an SW-28 rotor at 26 000 rpm for 2 hours at 4°C. The supernatant was then discarded, and the purified virus pellet was suspended in 0.6 mL sterile MEM. Titers typically are 3 to 5 x 10⁹ plaque-forming units per milliliter.

The following constructs have been described previously: the IE72 and IE84 expression plasmids; the CMV-reporter plasmid, MIEP–chloramphenicol acetyltransferase (CAT); the COX-1 and COX-2 expression vectors; the reporter plasmid COX-2–CAT (p102), which contains the nucleotides 582 to 101 of the COX-2 promoter upstream to a CAT reporter gene, or the deleted COX-2 promoter–CAT plasmid without or with 1 μg of the IE72 or the IE84 expression vector. Transfection was performed by lipofection, with N-[1-(2,3-dioleoloyloxy)propyl]-N, N, N-trimethyl ammonium methyl sulfate (DOTAP) reagent (Boehringer). Human 293 cells were grown in 10-cm dishes, transfected with COX-1 or COX-2 expression vectors (2 μg per dish) with lipofectamine according to the manufacturer’s instructions (Gibco/BRL), and harvested with a 10 mmol/L EDTA solution 24 hours later. They were then grown in 4-well chamber slides coated with human fibronectin (5 μg per cm²). After 24 hours they were infected with 5 multiplicities of infection (MOI) of CMV for 1 hour and, after removal of free virus, incubated with NS-398 for 1 hour. The drug was then removed, and the cells were prepared for detection of ROS by confocal microscopy as described above.

Immunoblots

SMCs were grown in 175 cm² flasks to 90% confluence, treated with drugs, infected with CMV, lysed, subjected to gel electrophoresis, and blotted. Steady-state protein levels of viral IE72 or human cellular COX-2 were assessed by immunoblotting with monoclonal anti-IE72 antibodies (6E1, Vancouver Biotech) as described elsewhere in detail. Polyclonal anti-COX-2 antibodies at a 1:1000 dilution and a chemiluminescence kit (Immun-star, Bio-Rad) were used for (protein) signal detection.

Viral Titer Assay and Cytopathic Effects

Human CMV (Towne strain) was passaged in our laboratory in HEL299 fibroblasts as described. Corneal SMCs were seeded in 48-well plates (15 000 per cm²) and, for immunocytochemistry of viral IE72 (72-kD IE CMV region-1 product), in 8-well glass chamber slides (Nunc) for 48 to 72 hours and then infected with CMV at 5 MOI. One hour after adsorption, free virus was removed and duplicate wells were treated with either vehicle, 2 mmol/L aspirin, or 2 mmol/L sodium salicylate. Infected SMCs and their growth media were sonicated 96 hours after infection and diluted 10-fold; viral titer was determined by plating aliquots of the sonicate on indicator fibroblasts. Cytopathic effects and plaque formation were assessed 3 to 10 days later by counting the number of foci of infected cells exhibiting cytomagic changes. Additional plates of SMCs were infected or mock-infected, treated with aspirin or sodium salicylate as described above, and harvested for cell counting 24, 48, and 96 hours later to ensure that changes in viral titer were not due to changes in cell number after infection.

Cyclooxygenase Inhibitor

To determine whether COX-1 and COX-2 contribute to the CMV-mediated induction of ROS, the following separate experiments were performed: infected SMCs were treated with (1) aspirin and indomethacin, 2 nonselective COX-1 and COX-2 inhibitors, (2) NS-398 and dexamethasone, a selective and a nonselective COX-2 inhibitor, respectively, and (3) sodium salicylate, which has minimal COX-inhibitory effects but is a ROS scavenger more potent than aspirin.

Results

Effects of Aspirin and Indomethacin on CMV-Induced ROS Generation

We have previously shown that CMV infection of HCSMCs generates intracellular ROS (Figure 1A, a and b), as assessed
by quantified confocal microscopic study of the fluorescence produced by the oxidation of DCFH-DA. Aspirin or indomethacin, 2 nonselective COX-1 and COX-2 inhibitors, diminishes CMV-induced ROS generation in a concentration-dependent manner (Figure 1A and Table). That this effect is not entirely due to inhibition of these enzymes is indicated by the finding that sodium salicylate also diminishes ROS generation (Figure 1A and Table). This compound has only minimal COX inhibitory activity but, like aspirin and indomethacin, is a potent ROS scavenger.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** A, Effect of aspirin, sodium salicylate, or indomethacin on endogenous ROS generation in CMV-infected SMCs: uninfected (a) and infected controls (b); aspirin (0.1 or 2 mmol/L) (c and d); sodium salicylate (0.1 or 2 mmol/L) (e and f); and indomethacin (2 or 20 μmol/L) (g and h). All cells (except in panel Aa) were infected with CMV at 5 MOI. Cells were infected with CMV, treated with drugs, and then exposed to DCFH-DA (5 μmol/L). The resulting fluorescence, indicative of ROS generation, was then assessed by confocal microscopy. The study was performed 3 times with similar results. Bar=200 μm. B, CMV-induced ROS generation is dependent on COX-2. Human 293 cells were transfected with expression plasmids containing empty vector DNA (a) or cDNA for COX-1 (b), cDNA for COX-2 (c), and cDNA for COX-2 plus treatment with 5 μmol/L NS-398 for 1 hour after infection (d). Twenty-four hours after transfection they were infected with CMV (5 MOI) for 1 hour and assessed for ROS, as in panel A. The study was performed 3 times with similar results. Bar=200 μm. C, CMV-induced ROS generation is dependent on COX-2: quantitative analysis. Relative fluorescence (RF) units from the experiments depicted in panel B were quantified, and 3 fields were analyzed for each bar depicted in the graph, as described in the Table. Cells transfected with plasmids encoding COX-1 or COX-2 but not infected with CMV exhibited little fluorescence.
### Role of COX-2 in CMV-Dependent ROS Generation

#### Effect of NS-398 or Dexamethasone

That COX-2 is involved, at least in part, in CMV-induced ROS generation, is indicated by the finding that the selective COX-2 inhibitors NS-398 and dexamethasone also decrease CMV-induced ROS generation (Table). NS-398 irreversibly inactivates COX-2, whereas dexamethasone decreases COX-2 mRNA accumulation.\(^{20,21}\)

#### CMV Induction of ROS Is Dependent on Expression of COX-2

To more directly examine the role of COX-2 in CMV-induced ROS activity, we transfected 293 cells, which have minimal COX-1 or COX-2 expression, with expression vectors containing either the gene encoding COX-2 or COX-1, or the empty vector.\(^{16}\) Using immunoperoxidase staining with a specific anti-COX-2 antibody, we found that transfection efficiency was 15% to 20% (not shown). ROS generation induced by CMV is strikingly enhanced in the cells transfected with COX-2 when compared with that in the cells transfected with COX-1, with the empty vector, or with the COX-2 vector but without CMV infection (Figure 1B and 1C). These results indicate that CMV-induced ROS generation is dependent, at least in part, on the presence of COX-2. Confirmation of this conclusion is demonstrated by the decrease in CMV-induced ROS generation produced by NS-398 in the COX-2-transfected cells (Figure 1B, d).

### Effects of IE72 and IE84 on Expression of COX-2–CAT in SMCs

We also found that the CMV IE gene products IE72 and IE84 have the potential to increase the transcription of the COX-2 gene (Figure 2A). We cotransfected the IE72 or IE84 expression plasmids into SMCs with either the COX-2 promoter–CAT reporter plasmid, which contains nucleotides –582 to +101 of the COX-2 promoter upstream to a CAT reporter gene, or with a deleted COX-2 promoter–CAT plasmid (–92 to +101), which lacks all inducible transcription factor–binding sites. IE72 and IE84 each transactivates the COX-2 promoter and exerts synergistic effects when cotransfected. The COX-2 promoter region that we used contains 2 NFκB and 3 SP-1–binding elements, among others. IE72 is known to regulate NFκB sites,\(^6\) and both IE72 and IE84 can activate SP-1–binding sites.\(^6\) The deleted reporter construct was unresponsive to IE72 and IE84.
Effect of Aspirin on Viral Titer
To determine whether the inhibition of ROS, NFκB, and IE72 translates into impairment of viral replication, we treated infected SMCs with aspirin immediately after infection and again after renewal of the medium at 48 hours after infection. We found that aspirin or sodium salicylate at 0.5 and 2 mmol/L doses decreased viral titer by 50% to 70%. This effect was not due to infection-related reduction in cell number, as shown by counting cells in another set of experimental wells (Figure 4).

PGE₂ Production After Human CMV Infection
At 3 to 6 hours after infection of SMCs with human CMV, large amounts of PGE₂ appeared in the medium. In contrast, uninfected cells released only small amounts of PGE₂. Release peaked at 3 to 6 hours and then declined at 12 hours after CMV infection of SMCs shown by immunoblotting. Samples were processed as above. The experiment was repeated once.

Discussion
The results of this investigation demonstrate that cyclooxygenases, and in particular COX-2, an IE mediator of inflam-
mation, are an important component of the pathway by which CMV infection of SMCs generate ROS and that ROS induction is prevented by both aspirin and indomethacin. ROS in turn activate NFkB, which regulates COX-2 and other inflammatory genes, notably intracellular adhesion molecule (ICAM)-1. We first found that aspirin and indomethacin inhibited ROS generation in a dose-dependent manner, as assessed by fluorescence and confocal microscopy (Figure 1A and Table). Because these agents inhibit both COX-1 and COX-2, we also determined the effects of the selective COX-2 inhibitors NS-398 and dexamethasone. Each of these agents markedly decreased the CMV-induced generation of ROS (Table). Thus, although we cannot exclude a role of COX-1, these results indicate that COX-2 is involved in CMV-induced ROS generation and in the synthesis of PGE2.

We more directly tested the conclusion that CMV induction of ROS is dependent on COX-2 by transfecting 293 cells (which, unlike SMCs, have minimal COX-1 or COX-2 expression), with expression vectors containing either the gene encoding COX-2 or COX-1. ROS were generated after CMV infection only in the cells transfected with the COX-2 expression plasmid, an effect abolished by treatment with the specific COX-2 inhibitor NS-398 and dexamethasone. Each of these agents markedly decreased the CMV-induced generation of ROS (Table). Thus, although we cannot exclude a role of COX-1, these results indicate that COX-2 is involved in CMV-induced ROS generation and in the synthesis of PGE2 (Figure 5).

We previously speculated that the generation of ROS can be viewed as a protective mechanism of the cell, which can contribute to the induction of apoptosis and thereby prevent the infecting virus from replicating and infecting neighboring cells and to the activation of NFkB, which mediates expression of cellular genes involved in the immune and inflammatory responses, such as ICAM-1. We also extracted RNA from infected SMCs and demonstrated induc-

Figure 5. HCMV infection stimulates cultured SMC-cyclooxygenase activity, as measured by PGE2 production. Viral activation of SMCs induces marked increases in PGE2 generation throughout an 8-hour time course, in contrast to control SMCs. The specific COX-2 inhibitor NS-398 attenuated PGE2 release in a concentration-related fashion. Data represent mean ± SD. A total of 10 experiments were performed with similar results.
thereby would also inhibit viral replication. We found by Western blotting analysis of infected SMCs that aspirin, sodium salicylate, or indomethacin inhibited IE72 expression by >50% (Figure 3). Moreover, both aspirin and salicylate modulated viral replication (Figure 4).

The effects of aspirin are caused, at least in part, by its capacity to inhibit COX-2. However, aspirin is a complex drug with multiple effects. In particular, it is known to be a potent ROS scavenger,19 and, therefore, part of the effects of aspirin we have observed in this investigation may be due to this activity. That the inhibition of the CMV-induced generation of ROS is not entirely due to inhibition of COX-2 is indicated by the findings that sodium salicylate also diminishes ROS generation. Also attributable to the direct ROS scavenger activity of aspirin is our finding that this drug inhibits the capacity of H2O2 to transactivate the MIEP (Figure 2B).

Our data suggest that aspirin, salicylate, and indomethacin have anti-CMV effects by directly scavenging ROS. We and others26 also found that aspirin and salicylate inhibit NFκB, which is critical for the activation of gene expression not only of CMV but also of cytokines, adhesion molecules, and other mediators of the inflammatory response in injured arteries. ROS are known to activate NFκB, and antioxidants inhibit this activation. Recent reports have shown that treatment of patients with probucol, a potent antioxidant, substantially reduced luminal narrowing after balloon coronary angioplasty, presumably at least in part by inhibiting NFκB.27

This is the first time that aspirin has been shown to have antiviral effects. Although relatively high concentrations of aspirin were needed to achieve these effects, such concentrations are attained in the plasma of patients treated for chronic inflammatory diseases such as arthritis.18 These findings raise the possibility that aspirin has a previously unrecognized therapeutic effect in various clinical situations, such as when it is administered to patients with viral infections as an antipyretic agent, to patients undergoing angioplasty as an antplatelet agent, and to patients with atherosclerosis to prevent thrombotic complications of the disease.

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References
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