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

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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 angioplasty1–4 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.5 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.6 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 antplatelet 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 IE gene product 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 optimal 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 10⁶ 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 MIEP-CAT plasmid (p105, 92 to 101), which lacks all inducible transcription factor binding sites. The polyclonal anti–COX-2 antibody was raised by T. Hla. Aspirin (acetylsalicylic acid) and sodium salicylate were purchased from Sigma, and NS-398 was obtained from Biomol.

Assessment of Intracellular Redox State
Intracellular ROS generation after CMV infection was measured as described in detail elsewhere. Briefly, cells were grown in 4-well chamber glass slides (Nunc), infected with CMV for 1 hour and treated with drugs for 1 hour after removal of the virus. Cells were incubated for 5 minutes with 5 μmol/L 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probes), a non-polar dye that diffuses into cells. The dye is then deacetylated, and the polar derivative becomes fluorescent only when oxidized by H₂O₂ or hydroxyl radicals. Fluorescence was monitored and recorded by laser-scanning confocal microscopy (Leica TC554D, Leica Lasertechnik) as described.

Cyclooxygenase Inhibitors
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.

Transfections and CAT Assays
Human coronary SMCs were grown in 10-cm dishes and transfected with 1 μg of the COX-2–CAT alone or cotransfected with 2 μg of the IE72 or IE84 plasmid or with 0.1 μg of the major IE CMV–promoter MIEP-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-dioleoyloxy)propyl]-N, N, N-trimethylammonium 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 (Immuno-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. Coronary SMCs were seeded in 48-well plates (15 000 per cm²) and, for immunocytochemistry of 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 cytomagical 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 Activity
Human coronary SMCs (HCSMCs) were plated in 24-well dishes for 48 hours and then infected with human CMV (5 MOI) for 1 hour. After removal of the virus, cells were treated for 1 hour with serum-free media containing 1, 5, or 10 μmol/L NS-398. The medium was then replaced with 1 mL of serum-free medium. The media supernatant was removed at 3, 6, and 12 hours and centrifuged at 3000 rpm for 10 minutes to remove cell debris; supernatants were flash-frozen in ethanol/dry ice and stored at −70°C until assay. Aliquots (50 μL) of collected samples were assayed for spontaneously released prostaglandin (PG) E₂ by Enzyme Immuno Assay as described by the manufacturer (Amersham) (Figure 5).

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. 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=500 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.10,21

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,17 with expression vectors containing either the gene encoding COX-2 or COX-1, or the empty vector.18 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 SP1–binding elements, among others. IE72 is known to regulate NfκB sites, and both IE72 and IE84 can activate SP1–binding sites.19 The deleted reporter construct was unresponsive to IE72 and IE84.

Effects of Aspirin on Activation of MIEP-CAT by H2O2 and IE72 in SMCs
IE72 transactivates, in an ROS-dependent manner, its own promoter, the MIEP, through NfκB sites.5 This step is critical for expression of downstream CMV genes.19 We found that aspirin inhibits, in a concentration-dependent manner, the MIEP transcriptional activity of IE72 (Figure 2B). This effect may be partly due to a decrease in ROS secondary to COX-2 inhibition; however, aspirin also inhibits the capacity of H2O2 to transactivate the MIEP (Figure 2B), indicating that another mechanism may be operative through a direct ROS scavenger effect.

Electrophoretic Mobility Shift Assay
We have previously shown by gel shift assay that CMV infection of SMCs activates NfκB, an effect that is inhibited by antioxidants such as N-acetylcysteine.5 Here we determined that 1 to 2 mmol/L aspirin inhibits CMV-induced NfκB activation in a concentration-related fashion (not shown).

Immunoblotting
The inhibitory effects of aspirin, sodium salicylate, and indomethacin on IE72 expression were confirmed by Western blot analysis (Figure 3). Both active CMV infection and reactivation of latent CMV leading to release of progeny follow a cascade of IE, early, and late events: activation of the MIEP, which leads to expression within 2 to 4 hours of the major IE protein IE72, a potent transcription factor for both viral and cellular promoters. IE72 then transactivates the MIEP through NfκB binding sites,7 which leads to expression of IE84. The latter protein is a transcription factor more powerful than the former, which activates the early viral promoter and multiple cellular genes to create a milieu favorable to progression of infection and packaging of viral progeny. Because IE72 is a critical component of the infectious cycle for both acute and latent CMV, interventions aimed at inhibition of IE72 are reasonable and advantageous. We demonstrate that aspirin, indomethacin, or sodium salicylate inhibits IE72 expression by >50% (Figure 3).

We further show that steady-state levels of the COX-2 protein accumulated at 3 to 12 hours after infection, whereas COX-2 levels in uninfected cells were undetectable (Figure 3A). This confirms availability of the enzyme for PGE2 synthesis and release (Figure 5).
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).

Figure 2. A, IE72 and IE84 are capable of transactivating the COX-2 promoter. SMCs were transfected with either the COX-2–CAT reporter plasmid (nucleotides −582 to +101) encoding the binding elements 2 NFκB, 3 SP-1, and one each, AP-2, C/EBP, Ets-1, and CRE or with the COX-2 mutant CAT reporter (all binding sites removed) and cotransfected with expression plasmids encoding either IE72 or IE84, or with both. Columns indicate the average of 3 independent experiments; error bars represent standard deviation of the means. B, Effect of aspirin (ASA) on activation of the CMV MIEP by H2O2 and by IE72. HCSMCs were transfected by lipofection with 0.1 µg of the CMV reporter plasmid MIEP-CAT or cotransfected with 1 µg of the IE72 expression plasmid. At 35 hours after transfection, SMCs were treated with ASA or vehicle for 1 hour. The drugs were removed, and for the H2O2 experiment, fresh medium containing 10 µmol/L H2O2 was added. Cells were harvested 12 hours later and processed for CAT activity. Shown are means of 3 separate experiments. *Statistical significance of P<0.03.

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Figure 4. Effect of aspirin (ASA) or sodium salicylate (NaSal) on viral titer. HCMV-infected coronary SMCs (5 MOI) were kept in drug-supplemented maintenance medium for 96 hours. Aliquots of sonicated SMCs at 1:10 dilutions in serum-free medium were plated on indicator cells (HEL299, ATCC), and cytopathic foci of cytomegalic cells (by morphology) were recorded 96 hours later.
PGE2 (Figure 5).

In CMV-induced ROS generation and in the synthesis 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, and (3) COX-2 activity by concentration-related inhibition of PGE2 release after treatment of the cells with the specific COX-2 inhibitor NS-398 (Figure 1B and 1C). We speculate that CMV-induced COX-2 activation is, at least in part, caused by NFκB activation.

We have 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 NFκB, 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 induction of ICAM-1 mRNA (by Northern blotting) at 3 to 12 hours after CMV infection, whereas uninfected cells had no detectable message (not shown). CMV appears to have adapted to the cellular defense mechanism involving the release of AA and activation of COX-2, a known mediator of inflammation, with subsequent generation of ROS and the resulting activation of NFκB, which regulates transcription of COX-2, the CMV promoter, and the ICAM-1 promoter. During latent infection, stimuli to host cells that activate NFκB also lead to activation of the viral promoter and to direct expression of viral IE genes. It is tempting to speculate that the virus has evolved to benefit from the cellular mechanism of ROS generation to activate its own genetic programs and to ensure frequent albeit abortive reactivation from latency.

We also found that the IE gene products IE72 and IE84 of CMV can transactivate the COX-2 promoter (Figure 2A). By increasing the expression and therefore the activity of COX-2, the virus facilitates the increase in ROS that follows infection and thereby further ensures that the intracellular environment is favorable to viral gene expression.

The IE protein IE72, a nuclear phosphoprotein, is expressed within a few hours after infection and then transactivates its own promoter, the MIEP, through multiple NFκB sites. Because IE72 synthesis is critical for the ensuing cascade of CMV gene expression in both acute infections and in reactivation from latency, interventions aimed at inhibiting this major IE CMV protein product would seem most beneficial. Because ROS are important for transactivation of the MIEP by IE72, we next determined whether aspirin interferes with this step of viral gene expression. By cotransfecting an IE72 expression plasmid and an MIEP-CAT reporter gene construct into human coronary SMCs, we found that aspirin inhibits, in a concentration-dependent manner, the transcriptional activity of IE72 (Figure 2B).

We previously showed that CMV infection of SMCs activates NFκB. This effect appeared to be dependent on ROS, as NFκB binding to its DNA was inhibited by antioxidants. In this investigation we found that NFκB/DNA binding in SMCs is inhibited by aspirin in a concentration-related fashion (not shown).

On the basis of these findings, we predicted that aspirin would attenuate IE72 expression after CMV infection and

![Figure 5](http://circres.ahajournals.org/)

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 SMC rates. 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.
CMV Infection Is Modulated by COX-2 in HCSMCs

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 antithrombotic agent, and to patients with atherosclerosis to prevent thrombotic complications of the disease.

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References

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