Aspirin Protects Endothelial Cells From Oxidant Damage Via the Nitric Oxide-cGMP Pathway

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Objectives—Aspirin is known to exert cytoprotection by presently unidentified mechanisms. This study investigates the involvement of nitric oxide (NO) in antioxidant cellular protection induced by aspirin.

Methods and Results—A 24-hour incubation with hydrogen peroxide markedly reduced viability of cultured endothelial cells. Preincubation with aspirin (3 to 30 μmol/L) protected endothelial cells from hydrogen peroxide–mediated toxicity and increased viability in a concentration-dependent fashion by up to 95% of control. This effect was specific in that other nonsteroidal anti-inflammatory drugs, such as salicylate or indomethacin, did not alter hydrogen peroxide toxicity. Aspirin-induced endothelial protection was abrogated in the presence of the NO scavenger PTIO (30 μmol/L) and the inhibitor of soluble guanylyl cyclase ODQ (1 μmol/L). Moreover, the L-arginine antagonist L-NMMA (25 μmol/L), but not its D-enantiomer, led to complete inhibition of aspirin-dependent cytoprotection. Correspondingly, aspirin enhanced NO synthase activity (citrulline formation) and intracellular cyclic GMP accumulation in endothelial cells. Protein expression of endothelial NO synthase remained unaffected in the presence of aspirin.

Conclusions—Our data suggest that endothelial NO synthase is a site of action of aspirin and that the NO/cyclic GMP system assumes a crucial function in mediating the cytoprotective action of aspirin. (Arterioscler Thromb Vasc Biol. 2003;23:1345-1351.)

Key Words: aspirin ■ nitric oxide ■ cyclic GMP ■ endothelium ■ antioxidant defense mechanism

A spirin reduces the incidence of thrombotic occlusive events such as myocardial infarction and stroke. This effect is considered to be attributable to the platelet inhibitory action of aspirin, which results from irreversible inhibition of platelet cyclooxygenase activity and thromboxane formation. Thromboxane is a potent agonist and crucial mediator of vascular smooth muscle contraction and platelet aggregation. Recently, however, a more direct effect of aspirin on the integrity of the vascular wall has been reported that comprises free radical scavenging properties of aspirin and its capacity to protect endothelial cells from the deleterious effects of hydrogen peroxide. Antioxidant effects of aspirin leading to the suppression of lipid peroxidation and reduced vascular tone have also been demonstrated in vivo in experimental animals and humans.

Although the mechanisms responsible for the observed aspirin-induced endothelial protection are largely unknown, we and others have found a very similar profile of cytoprotection evoked by nitric oxide (NO) or NO donors. Thus, NO as well as aspirin reduced the sensitivity of endothelial cells to hydrogen peroxide and other pro-oxidant agents in a time-dependent fashion. Both aspirin and NO donors induced long-term protective effects in endothelial cells that occurred after short periods of pretreatment and were sustained after washing out either agent. The similarity in the antioxidant actions of aspirin and NO led us to assume that NO might have a role as a downstream mediator in aspirin-dependent endothelial protection.

Our aim, therefore, was to investigate whether endothelial NO synthase (NOS) is a site of action for aspirin and contributes to the reduction of oxidant injury seen under the influence of this drug. Moreover, the present study explores a possible involvement of the NO-sensitive soluble guanylyl cyclase and the second messenger cyclic GMP (cGMP) in the action of aspirin.

Methods

Materials

FBS, cell culture media, and penicillin-streptomycin were obtained from Gibco. The chemiluminescence Western blotting kit and anti-rabbit peroxidase-conjugated secondary antibody were from Boehringer Mannheim (Mannheim, Germany). The cGMP enzyme immunoassay kit, phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), and 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) were purchased from Alexis Deutschland GmbH. L-arginine was from Amersham. YC-1 was kindly provided by Dr Johannes-Peter Stasch, Pharma Research Center, Bayer AG (Wuppertal, Germany). The polyclonal antibody against human endothelial NOS (eNOS) and all other chemicals were bought from Sigma (Deisenhofen, Germany).
Cell Culture
Vascular endothelial cells derived from bovine pulmonary artery were obtained as a cell line (ATCC CCL 209, batch No. F-11811 and 1336517) from the American Type Culture Collection.16,18 Bovine endothelial cells were maintained and subcultured in DMEM supplemented with 15% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Measurements of cGMP were performed in porcine aortic endothelial cells that are known to respond in a particularly sensitive manner to autocrine release of NO.19 These cells were isolated from different porcine aortas and characterized as described previously.18 They were maintained and subcultured (up to passage 3) in DMEM supplemented with 15% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were grown in a humidified incubator at 37°C (95% room air, 5% CO2). Expression of eNOS was measured in EA.hy 926 endothelial cells that were obtained from Dr Cora-Jean S. Edgell (Pathology Department, University of North Carolina, Chapel Hill, NC). This cell line was established as a model system for characterizing the regulation of eNOS and cultured according to published protocols.19 Rat fetal lung fibroblast cells (RFL-6) were from the American Type Culture Collection (ATCC CCL 192) and cultured as previously reported.20

Cell Viability Analysis
Endothelial cells were seeded at 2×10⁴ cells per well in 96-well microtiter plates in 100 µL of media containing 15% FBS. After a 48-hour incubation at 37°C, cells reached confluence and were incubated for 12 hours in the presence of aspirin or other nonsteroidal anti-inflammatory drugs. PTIO, ODQ, or N-monomethyl-D-arginine (L-D/NMMA) was added 15 minutes before aspirin. After washing out the previously added agents, hydrogen peroxide was given to the cells and incubation at 37°C was continued for 24 hours, followed by a cytotoxicity assay. Hydrogen peroxide was used at different concentrations between 0.5 and 1 mmol/L to compensate for varying sensitivities of cell cultures to oxidant injury and to achieve comparable degrees of cytotoxicity with each experiment. Cell viability was measured by staining with crystal violet and to additional by the lactate dehydrogenase assay (LDH assay; Boehringer, Mannheim) as previously described.17,21,22 The crystal violet assay allows the colorimetric assessment of the remaining viable cells after the incubation procedure. Cells were washed with PBS, fixed with methanol for 5 minutes, and then stained for 10 minutes with a 0.1% crystal violet solution. After 3 washes with tap water, the dye was eluted with 0.1 mol/L trisodium citrate in 50% ethanol for 10 minutes. Optical density at 630 nm was monitored on a microtiter plate reader (Biotek EL 311s). For the LDH assay, cells were plated and incubated as in the crystal violet assay. The supernatant was used to quantitate the LDH release of the cells spectrophotometrically at 490 nm according to the manufacturer’s protocol. Total cytotoxicity (100%) was standardized on LDH release after incubation with Triton X-100.

Measurement of Hydrogen Peroxide Decomposition
Hydrogen peroxide decomposition was measured with luminol-enhanced chemiluminescence and performed according to the previously published protocols using a Berthold LB9507 luminometer at 37°C.23,24 Cells were cultured and incubated with aspirin or vehicle in 6-well plates in analogy to the cell viability protocol. After adding hydrogen peroxide, samples were taken from the supernatant at different time points and were subsequently incubated with the chemiluminescence enhancer luminol (100 µmol/L) for 5 minutes. Chemiluminescence (relative light units) was measured with an integration time of 10 seconds. Control wells with luminol alone and luminol with cells as well as controls with or without hydrogen peroxide were included in each experiment. There was no significant light signal in the presence of lucigenin (50 µmol/L) instead of luminol.

cGMP Measurement
Cells grown to confluence in 6-well plates were washed twice with 2 mL of a balanced salt solution containing 130 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L CaCl₂, 5.5 mmol/L glucose, and 20 mmol/L HEPES-NaOH, buffered to pH 7.3. Cells were exposed for 10 minutes at 37°C to PTIO, ODQ L/D-NMMA, or vehicle in the balanced solution containing 0.5 mmol/L isobutylmethylxanthine. Aspirin, other nonsteroidal anti-inflammatory drugs, or YC-1 were added and the incubation was continued for another 10 minutes at 37°C. The final assay volume was 1 mL. Supernatants were aspirated, and after addition of ethanol and subsequent evaporation, cGMP levels were determined by an enzyme-linked immunoassay according to the manufacturer’s protocol (EIA kit, Cayman) and as outlined earlier.23

NOS Activity (Citricline Formation)
NOS synthesis was measured by a modification of a previously described technique.26,27 Cells grown to confluence in 35-mm culture dishes were incubated at 37°C for 30 minutes in 1.5 mL of HEPES buffer (pH 7.4) containing 0.25% albumin from human serum with or without the NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME, 1 mmol/L). Subsequently, cells were stimulated with aspirin in the presence of 10 µmol/L L-arginine and 3.3 µCl/mL L-[3H]-arginine. After 15 minutes, the reaction was stopped with cold PBS containing 5 mol/L l-arginine and 4 mol/L EDTA, and the cells were denatured with 96% ethanol. After evaporation, the soluble cellular components were dissolved in 20 mmol/L HEPES sodium salt (pH 5.5) and applied to 2-mL columns of Dowex AG50WX-8 (Na+ form). The radioactivity corresponding to the [3H]-citrulline content of the eluate was quantified by liquid scintillation counting. Agonist-induced [3H]-citrulline production was expressed in fmol/mg per well. Basal [3H]-citrulline synthesis was determined from the L-NAME–inhibitable radioactivity in unstimulated cells.

eNOS Protein Analysis
Endothelial cells were cultured in 150-mm dishes as described above. After a 12-hour incubation with control media, aspirin, or staurosporine, cells were washed and extracted as described previously.16,19,28 Protein (75 µg) was applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis (7.5% gels). After electrophoresis, protein was transferred to a nitrocellulose membrane, and a polyclonal antibody to human eNOS (Sigma) was used to identify eNOS protein content. Antigen antibody complexes were visualized with the horseradish peroxidase chemiluminescence system according to the manufacturer’s instructions (Boehringer, Mannheim).

Statistical Analysis
Results are expressed as mean±SEM. Data were analyzed by ANOVA and subsequently by Bonferroni’s correction for multiple comparisons. Differences were considered significant at P<0.05. All data were determined out of n=3 to 6 independent experiments at different days and cell passages. The values in the cell viability measurements correspond to the average of 6 samples, and all other experiments were performed at least in triplicate.

Results
Endothelial Cell Viability
Exposure of endothelial cells to hydrogen peroxide resulted in an evident reduction of cell viability (Figure 1). Pretreatment with aspirin (3 to 30 µmol/L) protected the cells from cytotoxicity in a concentration–dependent manner (Figure 1A). Aspirin-induced cytoprotection was completely abolished by the NO scavenger PTIO (Figures 1A and 1C). The NOS inhibitor L-NMMA abrogated cytoprotection by aspirin, whereas the D-enantiomer D-NMMA was without effect under these conditions (Figures 1B and 1C). Aspirin-induced endothelial protection was likewise reversed in the presence of the inhibitor of soluble guanylyl cyclase ODQ (Figures 1B and 1C).
A cytoprotective effect comparable to aspirin was observed when preincubating the cells with the membrane-permeable cGMP analogue 8-bromo cGMP (1 to 10 μmol/L) (Figure 2A) or the cAMP analogue 8-bromo cAMP (1 to 10 μmol/L) (Figure 2B).

Cytoprotection by aspirin was specific in that other nonsteroidal anti-inflammatory drugs such as salicylic acid, indomethacin, and diclofenac left hydrogen peroxide–induced toxicity unaltered (Figure 4A). Aspirin, other nonsteroidal anti-inflammatory agents, PTIO, L-NMMA, D-NMMA, or ODQ alone had no significant effect on cell viability under these conditions (not shown).

Hydrogen Peroxide Decomposition

Incubation of endothelial cells with hydrogen peroxide (1 mmol/L) led to a marked increase in photon emission as assessed by luminol-enhanced chemiluminescence. Pretreatment with aspirin (10 μmol/L) for 12 hours and subsequent media change did not alter the hydrogen peroxide–dependent light emission (Figure 3).

**cGMP Levels**

Aspirin (10 to 300 μmol/L) increased intracellular levels of the second messenger molecule cGMP in a concentration-dependent fashion. A significant cGMP formation already occurred at aspirin concentrations (30 μmol/L) that were effective in endothelial protection (Figure 5A). Additional experiments were performed at maximally effective concentrations of aspirin. Preincubation with the NO scavenger PTIO (30 μmol/L) led to complete inhibition of endothelial cGMP stimulation by aspirin (Figure 5A). In agreement with the results on cell viability, aspirin led to a specific elevation of cGMP levels that was not observed with other nonsteroidal anti-inflammatory drugs such as salicylic acid, indomethacin, or diclofenac (Figure 4B). YC-1, a direct, NO-independent stimulator of soluble guanylyl cyclase that is known to sensitize the enzyme toward its gaseous activator NO, produced an overadditive cGMP increase when given in combination with aspirin (Figure 5B). Under conditions of simultaneous incubation with aspirin and YC-1, L-NMMA only reversed the aspirin-dependent cGMP stimulatory effect,
pointing to the involvement of the l-arginine/NO pathway in the action of aspirin (Figure 5B). Moreover, in RFL-6 cells that are devoid of NOS activity but rich in soluble guanylyl cyclase, basal cGMP levels remained unchanged after an incubation with aspirin but rose to several fold over basal in a control experiment with glyceryl trinitrate, a NOS-independent donor of NO (Figure 5C).

**NOS Activity and Expression**

Measurements of NOS activity, determined as citrulline formation, were performed in protein extracts of endothelial cells as described by Bredt and Snyder and in the intact endothelial cell system. The basal citrulline formation was not altered by aspirin in the enzyme extracts (data not shown), whereas NOS activity increased significantly after direct incubation of endothelial cells with aspirin. The citrulline formation was completely depressed by the L-arginine antagonist L-NAME at 1000 μmol/L (data not shown for 300 μmol/L) (Figure 6A).

Enhanced NOS activity in the presence of aspirin was not associated with induction of eNOS protein expression (Figure 6). In a positive control experiment, staurosporine caused a marked elevation of eNOS protein levels (Figure 6).

**Discussion**

The beneficial cardiovascular effects of aspirin are generally attributed to its immediate platelet inhibitory function. However, accumulating evidence suggests that aspirin may have additional biological properties on the vasculature that contribute to the reduction of ischemic cardiovascular events in patients with hypertension and atherosclerosis. These possible nonplatelet-mediated effects include the attenuation of atherosclerosis attributable to inhibition of vascular smooth muscle cell proliferation, reduction in proinflammatory mediators, or improvement of endothelial dysfunction. Recent work by different groups has revealed that aspirin is capable of directly protecting the endothelium from the deleterious effects of oxidant stress. The underlying mechanisms have remained obscure.

The present study demonstrates that NO, which has long been known to improve endothelial dysfunction, is a crucial mediator of aspirin-induced endothelial cell protection. Pretreatment with aspirin caused a concentration-dependent increase in the surviving fraction of endothelial cells that were exposed to oxidant stress in the form of hydrogen peroxide. This cell-protective action of aspirin was completely abolished in the presence of the NO scavenger PTIO. A role for NO as effector molecule is additionally supported by our observation that incubation with aspirin results in enhanced accumulation of endothelial cGMP levels. The cyclic nucleotide cGMP is not only the second messenger of NO in many biological systems but has also been established as a sensitive marker of intracellular NO formation. That aspirin increases cGMP via NO and subsequent activation of soluble guanylyl cyclase is clearly demonstrated by a series of experiments using YC-1. YC-1 is a direct, NO-independent stimulator of soluble guanylyl cyclase that is known to sensitize the enzyme toward its gaseous activator NO. A simultaneous incubation of cells with YC-1 and aspirin produced an overadditive increase in cGMP that is typically seen when YC-1 is given in combination with submaximally effective concentrations of NO. ODQ is a selective inhibitor of soluble guanylyl cyclase, the cGMP-generating target enzyme of NO, and was found in the present study to abrogate cytoprotection by aspirin. These findings confirm the role of the NO/cGMP pathway in this
process and point specifically to cGMP as a causative mediator in antioxidant protection. Moreover, this assumption corresponds with the observed cytoprotective action of the membrane-permeable cGMP analogue 8-Br-cGMP. A similar endothelial protection could be demonstrated for the cAMP analogue 8-Br-cAMP, which has previously been reported as a possible mediator of cGMP-dependent cytoprotection in endothelial cells.44 It is noteworthy and in agreement with the results presented here that the stress proteins ferritin and heme oxygenase-1 have been identified as downstream mediators of NO-induced endothelial protection15,17,45 and were likewise shown to be inducible through aspirin.16,46

According to our findings, the enhanced activity of the NO/cGMP system under the influence of aspirin is attributable to a direct stimulatory effect of aspirin on eNOS. Thus, the inhibitor of NOS L-NMMA but not its D-enantiomer entirely blocked cytoprotection by aspirin, indicating involvement of enzymatic, L-arginine-dependent NO formation. Similarly, cGMP elevation in the presence of aspirin was attenuated by L-NMMA. Moreover, in RFL-6 cells that are devoid of NOS activity but rich in soluble guanylyl cyclase,20,47,48 basal cGMP levels remained unchanged after an incubation with aspirin. There was no effect of aspirin on eNOS protein levels in endothelial cells, precluding a stimulatory action on gene expression as possible explanation for the observed increase in NO-dependent cGMP accumulation. A genomic action of aspirin at this site seems also unlikely given the rapid onset of its effect on cellular cGMP levels.

Figure 5. A, Effect of aspirin on cGMP formation in endothelial cells. Inhibitory action of the NO scavenger PTIO. B, Effect of the direct, NO-independent activator of soluble guanylyl cyclase YC-1 and the NOS inhibitor L-NMMA on aspirin-dependent cGMP stimulation in endothelial cells. C, Comparison of the effects of aspirin and the organic nitrate GTN on cGMP formation in RFL-6 cells. Incubations and determination of intracellular cGMP levels were carried out as described in Methods. *P<0.05, treatment vs control (CON); #P<0.05, treatment with YC-1, aspirin, and L-NMMA vs YC-1 and aspirin. All data shown are mean±SEM of n=6 (A and B) and n=3 (C) independent observations with different cell passages and at different days. Incubations were performed in 6 separate cell culture wells (A) or in triplicate (B and C).

Figure 6. A, Influence of aspirin on NOS activity (citrulline formation) in endothelial cells. Inhibition through the L-arginine derivative L-NAME. B, Effect of aspirin and staurosporine (Stsp) on eNOS protein expression in endothelial cells. Experiments were carried out as described in Methods. *P<0.05, treatment vs control (CON); #P<0.05, treatment with aspirin and L-NAME vs aspirin alone. All data for A are shown as mean±SEM of n=6 independent observations in separate cell culture wells. Each incubation was performed in triplicate. B, Densitometric data are shown as mean±SEM of n=4 independent observations with different cell passages and at different days. One representative Western blot analysis is shown in the top panel.
However, aspirin significantly augmented citrulline formation from L-arginine, demonstrating enhanced NOS activity in the intact endothelial cell system. Therefore, it seems possible that aspirin may regulate eNOS activity posttranslationally by the availability of its substrate, L-arginine, or cofactors (eg, tetrahydrobiopterin or NADPH) or by protein-protein interactions, for example with caveolin or heat shock protein 90.49 Interestingly and in support of the data presented here, a previous study reported a stimulatory effect of aspirin on NO synthesis in neutrophils.50 Our study, however, is the first to establish eNOS as a site of action for aspirin and to demonstrate functional implications of this novel pathway.

The effects on endothelial integrity and the NO/cGMP system that we report here are specific for aspirin and not elicited by other nonsteroidal anti-inflammatory drugs such as diclofenac, indomethacin, or salicylate. This finding implies that it is the acetyl group within acetylsalicylic acid that confers the capacity to increase endothelial NO formation as well as antioxidant defense and that all other inhibitors of cyclooxygenase tested, including nonacetylated salicylate, do not fulfill the structural requirements necessary to activate NO. In a previous investigation, we have demonstrated a similar specificity of aspirin for the induction of the protective stress gene ferritin.16 Multiple other mechanisms of action have been proposed for aspirin beyond inhibition of COX.51 These include modulation of signaling molecules of the nuclear factor-oB signaling pathway, demonstrable in vitro.52 However, the concentrations used in most of these studies in vitro translate into toxic plasma concentration in vivo, some not compatible with life.53 With reference to the fact that in humans peak aspirin plasma concentrations of 4 µg/mL (which corresponds to ≈25 µM/L) can be obtained after the oral administration of a single 325-mg tablet,34 the effects of aspirin on endothelial function and cGMP accumulation were observed at therapeutically relevant concentrations. Clearly, more research is needed to additionally analyze the molecular mechanisms by which aspirin exerts its actions on endothelial NO and downstream targets.

In summary, we have demonstrated for the first time that aspirin is capable of activating the NO-cGMP signaling pathway in endothelial cells. Increases in NO and cGMP are causally related to antioxidant protection and improved integrity of the endothelium. Therefore, this novel pathway seems to be of functional relevance and may significantly contribute to aspirin-induced prevention of endothelial injury in cardiovascular disease, eg, during atherogenesis and other inflammatory processes. In the light of our investigations, activation of endothelial NO and cGMP formation could be an important mechanism by which aspirin, in addition to its platelet inhibitory action, protects against myocardial infarction, stroke, and death.

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