Aspirin Increases Ferritin Synthesis in Endothelial Cells
A Novel Antioxidant Pathway

Stefanie Oberle, Tobias Polte, Aida Abate, Hans-Peter Podhaisky, Henning Schröder

Abstract—Aspirin has recently been shown to increase endothelial resistance to oxidative damage. However, the mechanism underlying aspirin-induced cytoprotection is still unknown. Using cultured cells, the present study investigates the effect of aspirin on the expression of ferritin, a cytoprotective protein that sequesters free cytosolic iron, the main catalyst of oxygen radical formation. In bovine pulmonary artery endothelial cells, aspirin at low antithrombotic concentrations (0.03 to 0.3 mmol/L) induced the synthesis of ferritin protein in a time- and concentration-dependent fashion up to 5-fold over basal levels, whereas ferritin H (heavy chain) mRNA remained unaltered. Aspirin-induced cytoprotection from hydrogen peroxide toxicity was mimicked by exogenous iron-free apoferritin but not iron-loaded ferritin, demonstrating the antioxidant function of newly synthesized ferritin under these conditions. Ferritin induction by aspirin was specific in that other nonsteroidal anti-inflammatory drugs such as salicylic acid, indomethacin, or diclofenac failed to alter ferritin protein levels. Aspirin-induced ferritin synthesis was abrogated in the presence of the iron chelator desferrioxamine, pointing to an interaction of aspirin with iron-responsive activation of ferritin translation. Together, our results suggest induction of ferritin as a novel mechanism by which aspirin may prevent endothelial injury in cardiovascular disease, e.g., during atherogenesis. (Circ Res. 1998;82:1016-1020.)

Key Words: aspirin  ■ ferritin  ■ endothelial cell  ■ gene expression  ■ antioxidant defense mechanism

Aspirin is known to reduce the incidence of thrombotic occlusive events such as myocardial infarction and stroke.1 This effect is considered to be due to the platelet inhibitory action of aspirin, which results from irreversible inhibition of platelet cyclooxygenase activity and thromboxane formation.2 Thromboxane is a potent agonist and crucial mediator of vascular smooth muscle contraction and platelet aggregation.3 Recently, however, a more direct effect of aspirin on the integrity of the vascular wall has been reported in studies involving the antioxidant properties of aspirin and the capacity of aspirin to protect endothelial cells from the deleterious effects of hydrogen peroxide and, in particular, from iron-dependent oxygen radical formation.4–6

The mechanism responsible for the observed aspirin-induced endothelial protection is unknown. From the data published by Podhaisky et al.,7 it appears that cytoprotection by aspirin is a long-term effect that occurs after several hours of pretreatment. Therefore, a possible mechanism might be the induction of genes that protect cells from damage by reactive oxygen species. In recent studies, the induction of ferritin has been shown to provide marked antioxidant cellular protection by rapidly sequestering free cytosolic iron, the crucial catalyst of oxygen-centered radical formation via the Fenton reaction in biological systems.5,6 Thus, ferritin, which until then was thought to function merely as a “housekeeper” iron storage protein, has emerged as a critical and fast-acting endogenous cytoprotectant that plays an important role in cellular antioxidant defense mechanisms.5,10 Our aim, therefore, was to investigate whether ferritin is a potential site of action for aspirin and to characterize the effect of aspirin on the expression of ferritin in endothelial cells.

Materials and Methods

Materials

Bovine pulmonary artery endothelial cells (ATCC CCL 209) were obtained from the American Type Culture Collection. FBS, DMEM, and penicillin/streptomycin were obtained from GIBCO. Hydrogen peroxide was from Merck. The RNAeasy Total RNA-Kit was from Qiagen. The DIG DNA Labeling and Detection Kit, the Chemiluminescence Western Blotting Kit, and β-actin cDNA were from Boehringer Mannheim. The 530-bp Pst I ferritin H (heavy chain) cDNA fragment was a kind gift from Dr Gaetano Cairo (Centro di Studio sulla Patologia Cellulare, CNR, Milano, Italy).4 Apoferritin and all other chemicals were bought from Sigma.

Cell Culture

Endothelial cells were maintained and subcultured in DMEM supplemented with 15% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.11 The cells were grown in a humidified incubator at 37°C and 5% CO₂.

Ferritin mRNA Analysis

Ferritin mRNA content was analyzed in endothelial cells grown in 150-mm culture dishes after treatment with control media or aspirin for 6 hours. Total RNA was isolated with the RNAeasy Total RNA Kit. RNA (40 μg) from each sample was denatured and fractionated by 1% agarose-formaldehyde gel electrophoresis. RNA was visual-
ized by ethidium bromide staining, transferred to nylon membranes, and baked for 0.5 hours at 120°C. The membranes were hybridized with a random-primed digoxigenin-labeled cDNA probe for human ferritin H or β-actin according to the DIG System user’s guide (Boehringer Mannheim). Afterward, membranes were incubated with the chemiluminescent substrate CSPD (Boehringer Mannheim) and exposed to x-ray film for 2 hours. Equal loading of each lane with RNA was assessed by staining 18S and 28S rRNA with ethidium bromide and by using β-actin cDNA. Autoradiographs were quantified by computer-assisted videodensitometry (Eagle Eye II-system, Stratagene). Autoradiograms were scanned by densitometry, and the results were normalized to the ratio of ferritin/β-actin hybridization seen in control (untreated) cells.

**Ferritin Protein Analysis**

Endothelial cells were cultured in 6-well plates as described above. After a 6-hour incubation with control media, aspirin, salicylic acid, diclofenac, or indomethacin, cells were washed and extracted as described previously. Desferrioxamine was added to the cells 30 minutes before aspirin. Protein (100 µg) was applied to SDS-PAGE. After electrophoresis, protein was transferred to a nitrocellulose membrane, and a polyclonal antibody to human ferritin (Sigma) was used to identify ferritin protein content. Antigen-antibody complexes were visualized with the horseradish peroxidase chemiluminescence system according to the manufacturer’s instructions (Boehringer Mannheim). Quantification of ferritin induction was performed using computer-assisted videodensitometry (Eagle Eye II-system, Stratagene).

**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 aspirin, apoferritin, or ferritin was added. After a 6-hour incubation, hydrogen peroxide was added to the cells. Incubation was continued for 20 hours, followed by a cytotoxicity assay. Cell viability was measured by staining with crystal violet as previously described. This colorimetric test allows assessment of the remaining viable cells after the incubation procedure. After they were washed with PBS, cells were fixed with methanol for 10 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 1 ml of 1% trisodium citrate in 50% ethanol for 10 minutes. Optical density at 630 nm was measured using a microtiter plate reader (Biotek EL 311s).

**Results**

Aspirin increased ferritin protein synthesis in a concentration-dependent fashion: 2.4±0.3 (0.03 mm/L aspirin), 3.6±0.4 (0.1 mm/L aspirin), and 4.0±0.3 (0.3 mm/L aspirin) (significant for all values: P<0.05 for aspirin versus control, 2-tailed t test). The densitometric data are mean±SEM of 3 independent experiments and represent fold induction versus control (untreated) cells. A representative experiment is shown in Figure 1A. However, mRNA levels of ferritin H remained unaltered in the presence of aspirin (Figure 1B). Induction of ferritin protein levels by aspirin (0.3 mm/L) was time dependent: 1.7±0.2 (4 hours), 3.5±0.3 (6 hours), 3.9±0.4 (8 hours), and 4.6±0.3 (10 hours) (significant for all values: P<0.05 for aspirin versus control, 2-tailed t test). The densitometric data are mean±SEM of 3 independent experiments and represent fold induction versus control (untreated) cells. A representative experiment is shown in Figure 2A. Over the entire time course assessed, aspirin had no effect on ferritin H mRNA (Figure 2B). Ferritin protein induction by aspirin was specific in that other nonsteroidal anti-inflammatory drugs, such as salicylic acid, indomethacin, or diclofenac, failed to alter ferritin protein levels (Figure 3). In the presence of the iron chelator desferrioxamine, aspirin-enhanced ferritin synthesis was abrogated: 3.4±0.3 (0.3 mm/L aspirin) (P<0.05 for treatment versus control, 2-tailed t test), 0.9±0.1 (0.3 mm/L aspirin+0.1 mm/L desferrioxamine) (P=NS), and 0.9±0.2 (0.1 mm/L desferrioxamine) (P=NS). The densitometric data are mean±SEM of 3 independent experiments and represent fold induction versus control (untreated) cells. A representative experiment is shown in Figure 4. Preincubating the cells for 6 hours with iron-free apoferritin (1 mg/mL) but not with iron-loaded ferritin (1 mg/mL) reduced hydrogen peroxide-mediated cytotoxicity by 50%, thus mimicking a similar antioxidant effect of aspirin (0.03 mm/L, toxicity reduction by 57%) under these conditions (Figure 5). Apoferritin, ferritin, or aspirin alone had no significant effect on endothelial cell viability.

**Discussion**

The present study demonstrates, for the first time, that aspirin induces the synthesis of the antioxidant defense protein ferritin. Ferritin, in addition to serving as an iron reserve for the maintenance of vital cellular functions, sequesters iron that might otherwise catalyze damaging oxidative reactions. This cytoprotective antioxidant role of ferritin has remained largely hypothetical until it was shown that cells overexpressing this protein are more resistant to oxidative injury, whereas organisms lacking a functional ferritin gene are more sensitive to oxidative stress. Using cultured endothelial cells, we observed ferritin induction by aspirin at therapeutically relevant concentrations...
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being achieved in plasma during low-dose antithrombotic dosing regimens.\textsuperscript{18,19} Earlier studies have reported inhibitory effects of aspirin on nuclear factor-κB (NF-κB, a transcription factor)\textsuperscript{20–22} and on the regulation of genes such as nitric oxide synthase\textsuperscript{23,24} but only at rather high concentrations (2 to 20 mmol/L), which are hardly reached or maintained in plasma even after anti-inflammatory doses of aspirin. Therefore, and in contrast to NF-κB or NO synthase, ferritin appears to be a molecular target of aspirin with a particular potential for clinical significance. Moreover, ferritin induction by aspirin might explain earlier observations showing that aspirin is capable of directly protecting cultured endothelial cells from oxidant injury.\textsuperscript{6} Interestingly, aspirin-depen-

![Figure 2: Time-dependent effect of aspirin (ASA) on ferritin protein expression (A) and mRNA levels (B) of ferritin heavy chain (ferritin H) in endothelial cells. Incubations, protein isolation, Western blot analysis, RNA isolation, and Northern blot analysis were performed as described in "Materials and Methods." The data shown are from 1 representative experiment that was performed 3 times with similar results. CON indicates control (absence of aspirin).](image)

![Figure 3: Effect of aspirin (ASA), salicylic acid (SA), diclofenac (DIC), and indomethacin (IND) on ferritin protein levels in endothelial cells. Incubations and ferritin protein analysis were carried out as described in "Materials and Methods." The densitometric data shown are mean±SEM of 3 separate experiments. *P<0.05 for treatment vs control (CON), 2-tailed t test.](image)

![Figure 4: Effect of desferrioxamine (Des) on aspirin (ASA)-induced ferritin protein synthesis in endothelial cells. Incubations and ferritin protein analysis were carried out as described in "Materials and Methods." The data shown are from 1 representative experiment that was performed 3 times with similar results. CON indicates control.](image)

![Figure 5: Effect of apoferritin (APO), ferritin (FER), and aspirin (ASA) on hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})-mediated cytotoxicity in endothelial cells. Incubations were carried out as described in "Materials and Methods." *P<0.05 for treatment versus control (CON), 2-tailed t test. All data shown are mean±SEM of 6 independent observations in separate cell culture wells. The experiment was performed 3 times with similar results using endothelial cell cultures at different passages.](image)
aspirin, suggesting that free cytosolic iron is required for this effect and that aspirin increases ferritin synthesis by interacting with and possibly activating the well-known iron-dependent posttranscriptional upregulation of ferritin. Interestingly, the ferritin-inducing effect we report here is 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 activate ferritin synthesis and that all other inhibitors of cyclooxygenase tested, including nonacetylated salicylate, do not fulfill the structural requirements necessary to activate ferritin translation. Aspirin has been demonstrated to specifically interact with and show affinity toward iron or iron complexes, and it is therefore conceivable that aspirin accelerates dissociation of the ferritin repressor protein from ferritin mRNA, thus allowing ferritin translation. Clearly, more research is needed in order to further analyze the molecular mechanisms by which aspirin exerts its ferritin-inducing effect.

On the basis of the outcome of recent clinical studies, an increase in cellular iron levels has been suggested as a coronary risk factor that (via enhancing oxidative stress) promotes atherosclerosis and raises the incidence of myocardial infarction. Moreover, elevation of catalytically active iron followed by increased expression of ferritin has been observed in atherosclerotic lesions of patients with coronary heart disease, underlining the in vivo relevance of this antioxidant pathway. There has been dispute about the availability in vivo of uncomplexed iron as a Fenton catalyst and the existence of catalytically active ferrous iron in vivo. Other in vivo evidence for the relevance of free or loosely bound iron in promoting damage by radical formation comes from studies showing beneficial and tissue protective effects of metal-chelating agents such as desferrioxamine under pathological conditions involving oxidative stress.

Other in vivo evidence for the relevance of free or loosely bound iron in promoting damage by radical formation comes from studies showing beneficial and tissue protective effects of metal-chelating agents such as desferrioxamine under pathological conditions involving oxidative stress. In light of our investigations, activation of endogenous iron sequestration could be an important mechanism by which aspirin, in addition to its platelet inhibitory action, protects against myocardial infarction, stroke, and death. Since ferritin is thought to play a broader role in the cellular defense against oxidative injury under inflammatory conditions, it is conceivable that increased ferritin expression may also contribute in general to the anti-inflammatory action of aspirin.

In summary, we have demonstrated for the first time that aspirin at therapeutically relevant concentrations is capable of activating the synthesis of ferritin, a protein with cytoprotective and antioxidant properties. Although, on the molecular level, aspirin appeared to enhance iron-dependent ferritin translation, the exact mechanism of aspirin-dependent ferritin induction is not yet fully understood and is currently being investigated in our laboratory. However, with regard to the functional consequences and considering the comparatively low aspirin concentrations required to trigger ferritin synthesis, it can be concluded that this novel antioxidant pathway may significantly contribute to aspirin-induced prevention of endothelial injury in cardiovascular disease, eg, during atherogenesis and other inflammatory processes.

Acknowledgments

This study was supported by the Deutsche Forschungsgemeinschaft (Schr 2989/8-1+2). We would like to thank Petra Schwartz and Martina Heidler for excellent technical and secretarial assistance, respectively.

References


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Circ Res. 1998;82:1016-1020
doi: 10.1161/01.RES.82.9.1016

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