Aspirin protects endothelial cells from oxidative stress – possible synergism with vitamin E

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Abstract A 24-h incubation with hydrogen peroxide (0.65 mM) markedly reduced viability of cultured endothelial cells. Preincubation with aspirin (3–30 μM) protected endothelial cells from hydrogen peroxide-induced toxicity and increased viability in a concentration-dependent fashion by up to 64% of control. A similar protection was observed with D-α-tocopherol acetate (vitamin E, 3–30 μM). The cytoprotective effects of aspirin and vitamin E against hydrogen peroxide were overadditive suggesting different mechanisms of antioxidant action. In agreement with this, cytotoxicity induced by iron, the main catalyst of oxygen radical formation, was substantially reduced by aspirin but not vitamin E. These results show that aspirin protects endothelial cells from oxidative stress possibly via binding or chelation of free cytosolic iron. Moreover, a combination of aspirin and vitamin E might be useful for the prevention of endothelial injury in cardiovascular disease, e.g. during atherogenesis.

Key words: Aspirin; Vitamin E; D-α-Tocopherol acetate; Endothelial cells; Cytoprotection; Antioxidant

1. Introduction

Aspirin is widely used in the secondary prevention of thrombotic occlusive events such as myocardial infarction and stroke [1]. There is general agreement that the underlying mechanism of action is through irreversible inhibition of platelet cyclooxygenase activity and thromboxane formation, the latter being a potent agonist of vascular smooth muscle contraction and the aggregation of platelets [2,3]. While increased platelet activity in fact plays a crucial role during acute thrombus formation, it is the reduced integrity of the endothelial cell lining, i.e. endothelial damage, that facilitates adhesion of platelets and other blood cells to the vascular wall and can thus be considered a primary cause of atherogenesis and thrombosis [4]. Moreover, the intact endothelial layer serves as a selective barrier for plasma lipids and also has antithrombotic properties [4]. In addition to platelet inhibition, protection and preservation of the vascular endothelium may therefore be an important and equally effective approach to interfere with the initiating events of atherosclerosis and to prevent occlusive cardio- and cerebrovascular diseases.

Compelling evidence suggests that oxidative stress is a cardiovascular risk factor and contributes significantly to endothelial damage during atherogenesis. Besides oxidation of low-density lipoprotein, oxygen-centered radicals are capable of directly injuring the endothelium, e.g. when released by attached neutrophils or monocytes [5,6]. In this study we used an established in vitro model of free radical injury in the vasculature by exposing cultured endothelial cells to oxidative stress induced by incubation with hydrogen peroxide [7,8]. Our aim was to determine if, in addition to its well-known platelet inhibitory action, aspirin has a direct endothelial protective effect, i.e. is capable of increasing endothelial resistance towards oxidative damage. The cytoprotective effects of aspirin are compared to those of D-α-tocopherol acetate (vitamin E), an established antioxidant and radical scavenger.

2. Materials and methods

2.1. Materials

Bovine pulmonary artery endothelial cells (ATCC CCL 209) were obtained from the American Type Culture Collection, Rockville, MD, USA. Fetal bovine serum, Dulbecco's modified Eagle medium and penicillin-streptomycin were obtained from Gibco, Eggenstein, Germany. Hydrogen peroxide, ammonium iron(II) sulfate, and 8-hydroxyquinoline were from Merck, Darmstadt, Germany. Vitamin E (D-α-tocopherol acetate), aspirin and all other chemicals were purchased from Sigma, Deisenhofen, Germany.

2.2. Cell culture

Endothelial cells were maintained and subcultured in Dulbecco's modified Eagle medium supplemented with 15% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin [9]. The cells were grown in a humidified incubator at 37°C, 5% CO_2 and 95% air.

2.3. Incubation procedure

Endothelial cells were seeded at 2×10^4 cells/well in 96-well microtiter plates in 100 μl of medium containing 15% fetal bovine serum. Upon reaching confluence cells were preincubated for 8 h with aspirin or/and vitamin E. After the preincubation period, hydrogen peroxide or a freshly prepared mixture of ammonium iron(II) sulfate (50 μM) and 8-hydroxyquinoline (50 μM), the latter serving as iron transporter

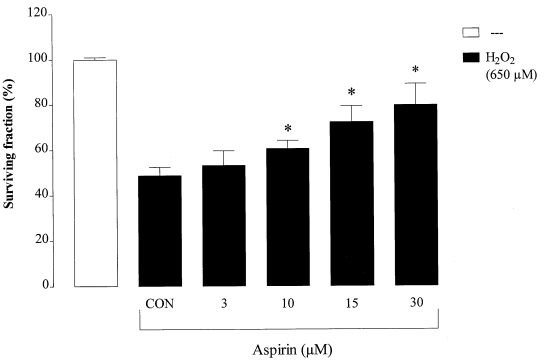

Fig. 1. Effect of aspirin on H_2O_2-induced cytotoxicity in endothelial cells. Incubations were carried out as described in Section 2. *$P<0.05$, aspirin vs. control (CON), two-tailed t-test. All data shown are mean ± S.E.M. of $n=6$ observations.

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Abbreviations: Vitamin E, D-α-tocopherol acetate

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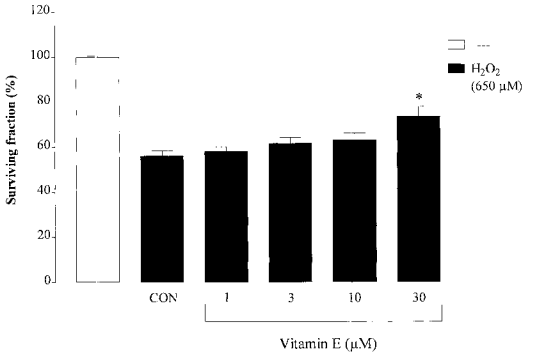

Fig. 2. Effect of vitamin E on H_2O_2-induced cytotoxicity in endothelial cells. Incubations were carried out as described in Section 2. $^*P < 0.05$, vitamin E vs. control (CON), two-tailed t-test. All data shown are mean ± S.E.M. of $n = 6$ observations.

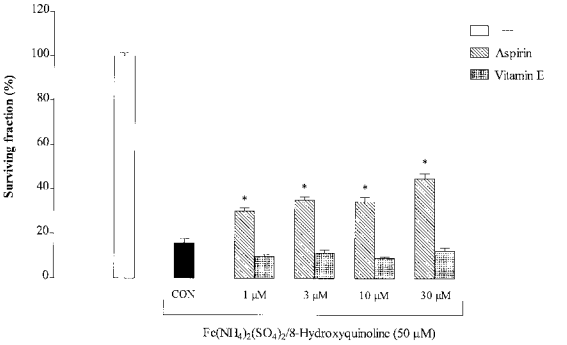

Fig. 4. Effect of aspirin and vitamin E on endothelial toxicity induced by ammonium iron(II) sulfate ($Fe(NH_4)_2(SO_4)_2$/8-hydroxyquinoline). Incubations were carried out as described in Section 2. $^*P < 0.05$, treatment vs. control (CON), two-tailed t-test. All data shown are mean ± S.E.M. of $n = 6$ observations.

across cell membranes [6], was added to the cells. Incubation was continued for 24 h, followed by a cytotoxicity assay.

2.4. Cytotoxicity assay

Cell viability was measured by staining with crystal violet as previously described [9–11]. This colorimetric test allows the assessment of the remaining viable cells and therefore does not discriminate between necrotic and apoptotic events. After washing with phosphate buffered saline, cells were fixed with methanol for 5 min and then stained for 10 min with a 0.1% crystal violet solution. Following three washes with tap water, the dye was eluted with 0.1 mol/l trisodium citrate in 50% ethanol for 1 h. Optical density at 630 nm was measured using a microtiter plate reader (Biotek EL 311s).

3. Results

In endothelial cells, treatment with hydrogen peroxide (650 μM) markedly reduced the number of viable cells (Fig. 1). Preincubation with aspirin (10–30 μM) protected endothelial cells from hydrogen peroxide-mediated cytotoxicity in a concentration-dependent manner and increased viability by up to 64% of control (Fig. 1). Vitamin E (3–30 μM) also tended to protect endothelial cells from oxidant damage (Fig. 2). However, a significant increase in the number of surviving cells (32% of control) was detected only at 30 μM vitamin E. Without preincubation, no significant protection by vitamin E and aspirin was detected suggesting that the agents need to penetrate (not shown). At subthreshold concentrations (20 μM), vitamin E had a potentiating effect on endothelial protection by aspirin. After co-incubation with vitamin E at non-protective concentrations (20 μM), endothelial protection by aspirin (10 μM) was increased by 50% (Fig. 3). When ammonium iron(II) sulfate/8-hydroxyquinoline (50 μM) was used as a cytotoxic agent [6], vitamin E had no effect on the surviving cell fraction (Fig. 4). In contrast, aspirin proved to be more potent in endothelial protection under these conditions as compared to hydrogen peroxide-mediated toxicity: at 1 μM and 30 μM aspirin, the surviving fraction of endothelial cells was increased by 88% and 290%, respectively. Aspirin, vitamin E, and 8-hydroxyquinoline alone had no significant effect on endothelial cell viability (not shown).

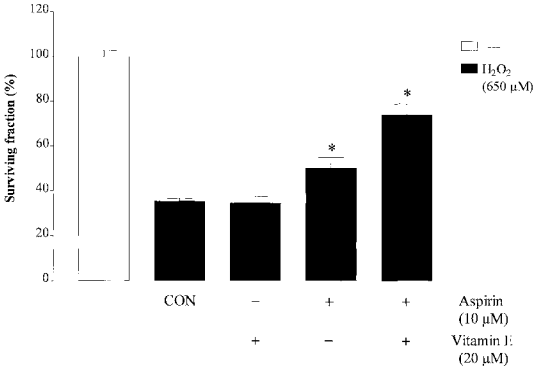

Fig. 3. Synergistic effect of aspirin and vitamin E on H_2O_2-induced cytotoxicity in endothelial cells. Incubations were carried out as described in Section 2. $^*P < 0.05$, treatment vs. control (CON), two-tailed t-test. All data shown are mean ± S.E.M. of $n = 6$ observations.

4. Discussion

The present study demonstrates protection against hydrogen peroxide-mediated endothelial toxicity by aspirin. This novel pharmacological action may be of therapeutic relevance since it was observed at concentrations that are in the range of systemic plasma aspirin levels during antithrombotic dosing regimens [12,13]. Our findings suggest that in addition to platelet inhibition, aspirin has the capacity to directly protect the endothelium from the deleterious influences of oxidative stress. The established antioxidant and radical scavenger vitamin E was also found to reduce cytotoxicity by hydrogen peroxide, which is in line with previous investigations [14,15]. However, when compared on a molar basis, aspirin turned out to be about 10-fold more potent in endothelial protection than vitamin E. Interestingly, aspirin has been shown to possess antioxidant and radical scavenging properties [16,17] which may explain the aspirin-induced endothelial protection against oxidative injury. Our findings are nevertheless surprising since aspirin-dependent effects on surface cells such as in gastric mucosa have been characterized as irritating or cytotoxic rather than protective [18]. Despite widespread concern that even under low-dose antithrombotic regimens aspirin may lead to suppression of endothelial prostacyclin, an antithrombotic and vasodilatory cyclooxygenase metabolite [12,19], according to our data aspirin appears to preserve overall endothelial integrity and thereby to actually support

the antithrombotic and antiatherogenic function of the endothelium under conditions of oxidative stress.

At subthreshold concentrations, vitamin E had a potentiating effect on endothelial protection by aspirin. After co-incubation with vitamin E at non-protective concentrations, endothelial protection by aspirin was substantially increased. This overadditive synergism indicates that despite sharing antioxidant properties, aspirin and vitamin E may have different sites and mechanisms of action. Further support for this assumption is provided by our observation that endothelial toxicity induced by the Fenton catalyst iron can be attenuated by pretreatment with aspirin but not vitamin E. Free iron (Fe^{2+}) is the crucial catalyst of oxygen-centered radical formation in biological systems and its removal or intracellular sequestration results in diminished cellular sensitivity to oxidant damage [5,6,8,20]. Aspirin is known to exhibit metal-binding abilities [16] and may thus withdraw iron ions from the site of oxygen radical formation. In agreement with this, we found that compared to hydrogen peroxide-mediated cell injury, aspirin was 10-fold more potent as a cytoprotective agent when toxicity was induced by exogenous ferrous sulfate possibly reflecting the high affinity between iron and aspirin. In contrast to aspirin, vitamin E acts mainly as a chain-breaking radical scavenger in biological membranes [5] which might explain the overadditive cytoprotection (potentiation) that occurs when combined with metal-binding aspirin. Of course, additional interactions such as facilitated membrane penetration of aspirin in the presence of vitamin E may also contribute to the observed synergism.

Together, our results demonstrate direct endothelial protection from oxidative damage by aspirin at antithrombotic concentrations. Aspirin-induced cytoprotection was markedly increased in the presence of vitamin E. Therefore, a combination of aspirin and vitamin E might be useful for the prevention of endothelial injury in cardiovascular disease, e.g. during atherogenesis.

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