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Aspirin inhibits human coronary artery endothelial cell proliferation by upregulation of p53

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Abstract

Aspirin (acetylsalicylic acid, ASA) is effective in the primary and secondary prevention of vascular events. This effect is mediated in large part by platelet inhibition; however, non-platelet-mediated effects may also be relevant in the overall efficacy of ASA. We determined the effect of ASA on the synthesis of DNA and total proteins in cultured human coronary endothelial cells (HCAECs). Fourth generation HCAECs were cultured and treated with ASA and rate of synthesis of DNA and total proteins was determined by incorporation of [^{3}H]thymidine and [^{3}H]proline, respectively. ASA inhibited DNA synthesis by 50% at a concentration of 1 mM and protein synthesis by 50% at a concentration of 2 mM. The inhibitory effect of ASA was observed as early as 2 h after treatment of HCAECs. The inhibition of DNA and protein synthesis could be reversed within 24 h after removal of the drug from the culture medium. Indomethacin also inhibited DNA and protein synthesis. Western blot analysis revealed that the expression of p53 protein was increased after treatment of the cells with ASA. These observations indicate that ASA decreases endothelial cell proliferation through cell cycle arrest mediated by enhanced p53 expression. Arrest of endothelial proliferation and activation may be an important mechanism of thes beneficial effect of ASA in acute coronary syndromes.

Keywords: Aspirin; Cell cycle; Cyclooxygenase; Endothelial cells

Aspirin (acetylsalicylic acid, ASA) is widely used in the primary and secondary prevention of vascular disease [1]. The anti-inflammatory effect of salicylates is believed to complement its platelet inhibitory effect and is believed to be due to the inhibition of cyclooxygenase, resulting in decreased thromboxane A_2 production [2]. Aspirin inhibits cyclooxygenase by acetylating the serine residue in the active site of the enzyme [3,4].

Although the major beneficial effect of ASA is due to its inhibitory action on platelet aggregation, there is emerging evidence that other effects of ASA on cells other than platelets may be equally important [5]. Bernhardt et al. [6] have reported that high concentrations of ASA can inhibit smooth muscle cell proliferation. Marra et al. [7] have shown that ASA inhibits the proliferation of smooth muscle cells through inhibition of cyclin-dependent kinases, which hyperphosphorylate the retinoblastoma protein. Other studies suggest antiarrhythmic effect of ASA in platelet-depleted dogs subjected to coronary occlusion [8].

We investigated the effect of ASA in cultured human coronary artery endothelial cells (HCAECs). Our studies show that aspirin strongly inhibits the synthesis of DNA and total protein without affecting the viability of the cells. These effects of ASA may play an important role in the tissue protective effects during acute myocardial ischemia by inhibiting the formation of deleterious cytokines and inflammatory molecules.

Materials and methods

Reagents. The culture medium for endothelial cells was obtained from Clonetics. Trypsin, acetyl salicylic acid, and indomethacin were obtained from Sigma Chemical ^{3}H-labeled thymidine was purchased

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from Perkin–Elmer Life Sciences and L-[^{3}H]proline was purchased from American Radiolabeled Chemicals.

Endothelial cell culture. The initial batch of HCAECs was obtained from Clonetics. The cells were purely based on morphology and staining for factor VIII antigen. The cells were negative for α-actin smooth muscle expression. These cells were cultured in endothelial cell basal medium supplemented with (per 500 ml) human epidermal growth factor 5 ng, hydrocortisone 5 mg, gentamicin 25 mg, amphotericin 5 μg, bovine brain extract 6 mg, and fetal bovine serum 25 ml. For experiments they were cultured in 12 well plates and used for the experiment when they were about 75% confluent. Aspirin and indomethacin were dissolved in 95% ethanol to make stock solutions. No more than 5 μl of the stock solutions was added per ml of the culture medium in the plates. The control plates were treated with 5 μl ethanol. The cells were used between fifth and tenth passages for all the experiments.

[^{3}H]Thymidine and [^{3}H]proline incorporation. To measure the rate of synthesis of DNA and protein, the cells were incubated with 0.5 μCi/ml ^{3}H-labeled thymidine or proline for 4 h. At the end of this incubation the monolayers were washed three times with phosphate-buffered saline and treated with 0.5 ml of 10% trichloroacetic acid for 10 min. The cells were then washed three times with 90% ethanol and lysed with 0.5 ml of 0.2 M NaOH. The lysates were transferred to scintillation vials containing 5 ml of ScintiSafe scintillation fluid and radioactivity was determined.

Western Blot analysis for p53. The cell monolayers were washed three times with PBS and lysed with RIPA buffer containing phenylmethylsulfonyl fluoride and aprotinin as protease inhibitors. The cell lysates (10 μg total protein) were denatured and subjected to 7.5% SDS–PAGE. The proteins were transferred to nitrocellulose membranes by electroblotting. The membranes were soaked in a blocking solution containing PBS with 4% non-fat dried milk and 0.05% Tween 20 for 1 h at room temperature. The membranes were incubated with p53 monoclonal antibodies (Santa Cruz Biotechnology) for 2 h and then with peroxidase conjugated goat anti-mouse secondary antibodies for 2 h at room temperature. The bands corresponding to p53 were detected using chemiluminescence reagent (Amersham).

Data analysis. All data represent means of at least three independently performed experiments. Data are presented as means ± SD.

Results

The treatment of endothelial cells with ASA inhibited the synthesis of DNA and total protein as assessed by the incorporation of [^{3}H]thymidine and [^{3}H]proline (Fig. 1). These effects of ASA were concentration-dependent. The synthesis of DNA was inhibited by 50%, 72%, and 95% by 1, 2, and 5 mM ASA, respectively. The inhibition of protein synthesis by the corresponding concentrations of ASA was 27%, 48%, and 70%.

The cell treated with ASA did not show any evidence of cytotoxicity, such as change in shape or detachment (data not shown). The total cellular protein concentration was slightly decreased in the ASA treated cells (25.6 ± 1.0 μg/well in the controls vs. 22.0 ± 1.1 μg/well in ASA treated cells, P-NS, $n = 3$).

In order to study the time course of the inhibition of DNA and protein synthesis by ASA, the cells were incubated with 5 mM ASA for 2 and 6 h and then the rates of incorporation of [^{3}H]thymidine and proline were determined. As shown in Fig. 2, the DNA and protein synthesis rates decreased by 47% and 56% in 2 h, and by 78% and 72% in 6 h, respectively. These data show that the inhibitory effect of ASA on DNA and protein synthesis is rapid.

To examine if the effect of ASA on DNA and protein synthesis is reversible, the cells were treated with 5 mM ASA for 24 h, washed, and then incubated in fresh medium without ASA for another 24 h. As shown in Fig. 3, ASA inhibited DNA and protein synthesis by 95% and 70%, respectively. After removal of ASA from the culture medium, the DNA synthesis increased to about 70% of that in the untreated cells and the protein synthesis went up to 85% of the control. These results show that ASA did not result in any permanent injury to the cells.

In the next set of experiments, the effect of indomethacin, another cyclooxygenase inhibitor, was studied. The data presented in Fig. 4 show that indomethacin also inhibited the synthesis of DNA and protein in a concentration-dependent manner. At a concentration of 0.25 mM the DNA synthesis was inhibited by 95% and the protein synthesis was inhibited by 80%.

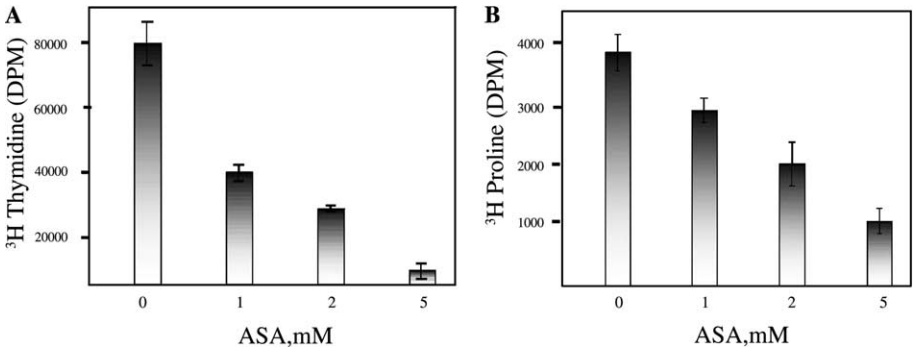

Fig. 1. Effect of ASA on DNA and total protein synthesis in HCAECs. The endothelial cells were grown in 12 well plates until they were about 75% confluent. The cells were treated with indicated concentrations of ASA for 24 h and then incubated with 0.5 μCi [^{3}H]thymidine or [^{3}H]proline per ml of medium for 4 h. At the end of this period the medium was removed and the cells were washed three times with phosphate-buffered saline and treated with 10% trichloroacetic acid for 10 min. The cells were then washed three times with 90% ethanol and dissolved in 200 μl of 0.2 N NaOH. The samples were transferred to scintillation vials and the radioactivity was determined. The data are averages of triplicate samples processed separately. The error bars indicate SD.

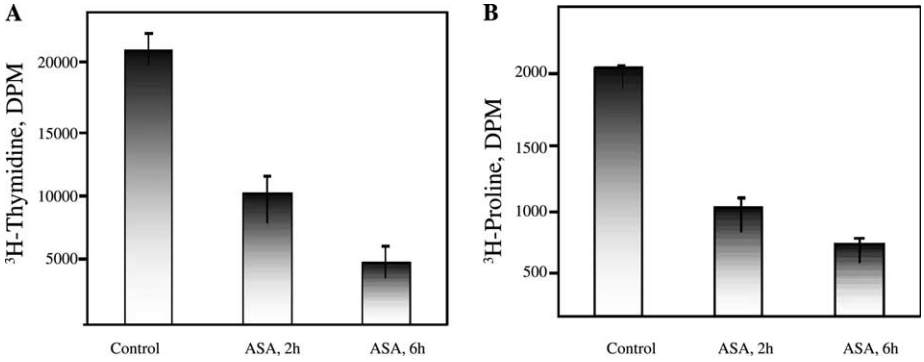

Fig. 2. Time course of the inhibitory effect of ASA in endothelial cells. The cells were cultured as described in the legend for Fig. 1 and treated with 5 mM ASA for the indicated time periods, before measuring the DNA and protein synthesis rates. The data are means of triplicate assays with SD shown as error bars.

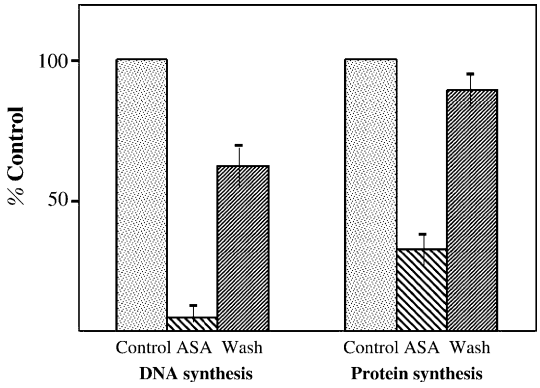

Fig. 3. Reversibility of the inhibition of DNA and protein synthesis by ASA after removal of ASA. The cells were cultured as described in the legend for Fig. 1 and treated with 5 mM ASA for 24 h. At this point one set of wells was used to measure the rate of DNA and protein synthesis. Another set was washed three times with the culture medium and incubated with ASA free medium for 24 h and then DNA and protein synthesis rates were measured. The data shown are means of triplicate analyses and the error bars are SD.

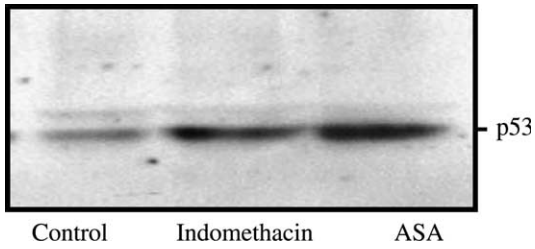

Fig. 5. Effect of ASA and indomethacin on p53 protein expression. The endothelial cells were treated with 5 mM ASA or 0.2 M indomethacin for 24 h. The methods for preparing cell extracts and Western blot analysis are described in Materials and methods. This Western blot is representative of three separate blots.

In order to examine if ASA has any effect in the regulation of cell cycle, we assessed the expression of p53, a tumor suppressor protein that regulates $p21^{Waf1}$, which in turn inhibits cyclin-dependent kinases. Western blot analysis showed that the expression of p53 protein was significantly increased in the cells treated with ASA, strongly suggesting that the inhibitory effect of ASA on DNA synthesis is mediated through p53 (Fig. 5).

Discussion

In the present study, we show that ASA inhibits DNA and protein synthesis in HCAECs. This effect

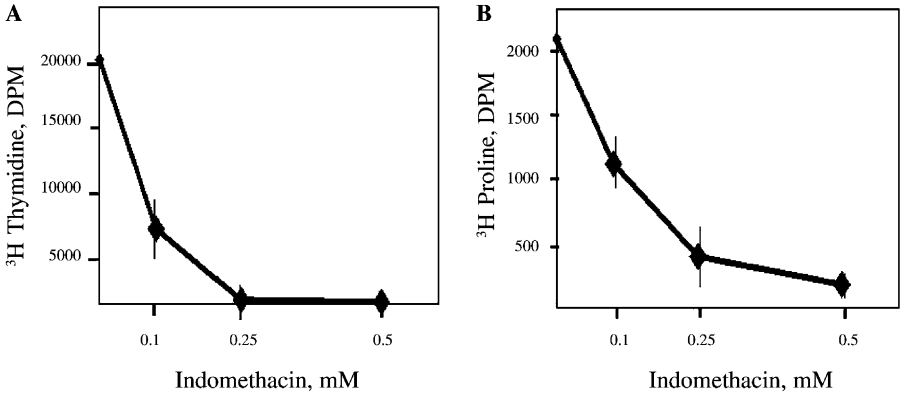

Fig. 4. Effect of indomethacin on DNA and total protein synthesis in endothelial cells. The cells were cultured as described in earlier figures and treated with indicated concentrations of indomethacin for 24 h and then the rates of incorporation of [^{3}H]thymidine and [^{3}H]proline were measured. The values shown are averages of triplicate independent analyses and the error bars indicate SD.

appears to be mediated through the upregulation of p53 expression, which is known to regulate the expression of $p21^{Waf1}$, one of the inhibitors of cyclin-dependent kinases (Cdks) [9]. Activation of Cdks is important in the hyperphosphorylation of retinoblastoma protein, which activates the transcription of several genes required for the progression of cell cycle [10].

Our data show that two different cyclooxygenase inhibitors, ASA and indomethacin, have strong inhibitory effect on DNA and protein synthesis in HCAEC. There are significant differences between smooth muscle cells and endothelial cells in terms of their sensitivity to ASA. We found that ASA inhibited DNA synthesis by about 50% at 1 mM concentration and by 95% at 5 mM concentration in HCAECs. It is of note that Marra et al. [7] observed that 1 mM ASA inhibited DNA synthesis by only 10% and 5 mM ASA inhibited DNA synthesis by about 70% in vascular smooth muscle cells. Indomethacin had no effect on cell proliferation in smooth cells, but it strongly inhibited DNA and protein synthesis in HCAECs. Thus it appears that the anti-proliferative effect of ASA is related to the inhibition of cyclooxygenase in the HCAECs, and the effect is much more potent than that in the smooth muscle cells. The concentration of ASA required for the inhibition in the in vitro studies may not be far from the pharmacological levels because the plasma concentration of salicylate can be in the millimolar range during ASA therapy [11]. Mason and Winer [12] have shown that plasma levels of salicylate can reach 0.3 mM after administration of 640 mg of aspirin.

Vascular endothelium is constantly exposed to inflammatory factors, such as cytokines and oxidized lipoproteins. Atherosclerosis in general reflects a low-grade inflammatory process and atherosclerotic plaques that are prone to rupture and result in acute coronary syndromes reveal an intense inflammatory state [13,14]. ASA is a cornerstone of therapy in acute coronary syndromes [1,5]. This agent has been shown to reduce atherosclerosis-related events in a multitude of clinical studies [15]. A number of studies show that ASA restores endothelial vasodilator properties [16–18]. Noon et al. [16] reported that in hypercholesterolemic subjects forearm vasodilation in response to acetylcholine is impaired and can be partially restored after addition of ASA. Similar observations were made in patients with coronary atherosclerosis [17]. Kharbanda et al. [18] have recently reported a protective effect of ASA against the endothelial, dysfunction.

Although most of the benefits of ASA have been ascribed to its anti-platelet aggregatory properties, non-platelet-mediated effects are also thought to contribute to its salutary effect [5]. From our observation of transient quiescence of endothelial cells, it is tempting to postulate that the protective effect of ASA may relate, at least in part, to its effect on hyperactive endothelium in response to a variety of cytokines and other inflammatory signals. Since the effect of ASA on the synthesis of DNA and proteins is reversible, this drug may not have any harmful effect on the metabolism and maintenance of the endothelium as the drug is metabolized rapidly.

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