Antithrombogenic Endothelial Cell Defense

Basal Characteristics in Cultured Endothelial Cells and Modulation by Short-term and Long-term Exposure to Isosorbide Nitrates

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The antithrombogenic endothelial cell defense (ATECD) describes the properties that enable the endothelium to prevent circulating blood platelets from adhering to, or aggregating on, the vascular wall. ATECD was investigated in an experimental model in which bovine passage 0 cultured endothelial cells (EC) were incubated with aggregating platelets and autologous plasma in a computer-operated aggregometer-like device. A maximal platelet aggregation required 150 × 10⁻⁹ M adenosine diphosphate (ADP) to be present in ECs. A 5-minute coincubation for ECs and platelets was found to be adequate in evaluating the maximal ATECD value. By increasing the EC number in the aggregation suspension, platelet aggregation was progressively inhibited through a sigmoid curve (50% inhibition of aggregation required 2 × 10⁴ EC). Pharmacologic modulations of ATECD by isosorbide dinitrate (ISDN) + 2-isosorbide mononitrate (2-ISMN) + 5-isosorbide mononitrate (5-ISMN) were investigated under experimental conditions reflecting either an acute nitrate effect (platelet + control ECs + drug + ADP) or a chronic effect (platelet + 5-day nitrate-treated ECs + ADP). Under acute circumstances, ISDN antiplatelet activities were profoundly magnified by ECs. Aggregation was fully arrested with 5 × 10⁻⁵ M ISDN and an EC number of 2 × 10⁴, whereas the same ISDN concentration alone induced 30% inhibition of control aggregation. In contrast, there were no significant changes in platelet aggregation whether incubation was done in the presence or absence of 2-ISMN or 5-ISMN, ISDN metabolites. Long-term exposure of ECs to isosorbide nitrates (ISN) resulted in increased acquired EC changes in ATECD. 5-ISMN was a poor antiplatelet agent but was capable of counteracting ISDN effects on ATECD. Under chronic circumstances, the overall ISN effect was a stimulation of ATECD, but the final effect was lower than that expected from the summation of individual ISN effects. Such an endothelium-dependent ISDN (and perhaps 2-ISMN) antiplatelet activity is likely to explain why ISDN inhibits platelet activity in vivo, while in vitro, ISDN fails to elicit such platelet aggregation inhibition unless suprapharmacologic ISDN concentrations are used. It is suggested that pharmacologic modulation of ATECD may be a new antithrombotic therapy. (Circulation Research 1987;60:612–620)

Organic nitrates are widely used in the treatment of vascular heart diseases, mainly because of their vasodilation properties. Aside from these vasodilation properties, which trigger the hemodynamic effects, many of the nitric-oxide–containing vasodilators, including isosorbide dinitrate (ISDN), have been reported to inhibit platelet aggregation as well as to prolong bleeding time in human beings. However, the biologic relevance of such antiplatelet properties has been hampered by conflicting results reported about the efficiency of organic nitrates to inhibit in vivo platelet activities on the one hand and, on the other hand, by a marked disparity between nitrate concentrations that inhibit platelet aggregation in vitro and therapeutic plasma levels obtained in vivo, the former being several orders of magnitude higher than the latter.

In concurrence with others, we have demonstrated that ISDN is a potent inhibitor of adenosine diphosphate (ADP)-induced platelet aggregation both in vivo and in vitro, while having far less effect on arachidonic acid-, collagen-, and epinephrine-induced aggregation in vitro. This restricted inhibition of platelet activities by ISDN may explain, at least in part, why in previous studies the absence of changes in platelet aggregation has been noted under organic therapy. To explain why the minimal nitrate concentration inhibiting platelet aggregation is much lower in vitro than in vivo, it has been proposed that organic nitrates may stimulate the production of prostacyclin (PGI₁), an antiaggregating and vasodilatory substance generated by the endothelium, which in turn decreases vascular tone and inhibits platelet activity. It is now generally acknowledged that the stimulating effect of nitrates on PGI₁ production is not likely to account for the vasodilation properties of these drugs. Alternatively, we demonstrated the biologic relevance of a second hypothesis—i.e., one or several ISDN metabolite(s) participate(s) in the in vivo ISDN antiplatelet effects and not solely the drug itself—by showing that 2-isosorbide mononitrate (2-ISMN) is a potent agent of platelet aggregation compared with ISDN and 5-isosorbide mononitrate (5-ISMN). However, the overall isosorbide nitrate (ISN) antiplatelet activities still cannot account for the in vivo ISN platelet effects.
In an attempt to account for the knowledge gap concerning in vivo vs. in vitro ISN antiplatelet activities the present studies were undertaken based on a hypothesis elaborated in recent work on blood vessel wall biology and pharmacology. This hypothesis emphasizes the importance of the interaction of endothelial cells with platelets and other cells (e.g., smooth muscle cells and monocytes) in processes contributing to the physiologic and pharmacologic regulation of vessel blood hemostasis and vasomotoricity. Like the vascular endothelial cells, nitrogen-oxide-containing vasodilators possess a bifunctional nature, making them capable of inhibiting platelet activity and of eliciting a diminution of vascular tone.

We have developed an in vitro model adapted from Curwen et al. in which bovine endothelial cells are coincubated with human platelets and autologous plasma in a computerized aggregometer-like device. The properties of the antithrombogenic endothelial cell defense (ATECD), although not precisely identified, enable the vascular endothelial lining to prevent circulating blood platelets from adhering to, or aggregating on, the vascular wall. Therefore, we investigated the possibility that in this experimental model, ISNs stimulate ATECD in an acute pharmacologic manner and/or amplify the intrinsic ATECD after long-term exposure of endothelial cells in culture to ISN.

Materials and Methods

Platelet Collection and Processing

Platelets were obtained from normal, healthy men or women volunteers with no prior evidence of liver or hematological disease and no exposure to aspirin-like drugs or other medication for at least two weeks prior to venipuncture as previously described. Informed consent was obtained. Blood samples were drawn from the midstream of a brachial vein by careful venipuncture after stasis of less than 10 seconds, using a siliconized 18-gauge needle. The first 2–3 ml of blood were discarded, and the subsequent 50 ml were collected in tubes containing citric acid/trisodium citrate (0.11 M) in a ratio of 1 part citrate for each 9 parts blood. Platelet-rich plasma (PRP) was prepared by centrifugation of blood at 150g for 20 minutes at 16°C. After the PRP was withdrawn, platelet-poor plasma (PPP) was prepared by centrifuging the remaining mixture at 6,000g at 16°C for 15 minutes. Platelet counts were determined with a Coultronics Thrombo-Counter. PRP was then diluted with autologous PPP until the platelet count was in the range of 300 × 10⁶ platelets/l. Platelet counts in fresh PRP were above this range.

Endothelial Cell Culture — Cell Harvesting

The preparation of endothelial cells (EC) from bovine arteries was performed following the method of Johnson, and ECs were identified according to the method of Schwartz. Bovine carotid and femoral arteries (6–8 fragments, 10–20 cm long) were collected at a local slaughterhouse from freshly killed adult animals. All vessels were carefully dissected free of adhering connective tissue, extensively rinsed in basal essential medium (BEM), and placed on moist, sterile gauze in 100-mm tissue culture petri dishes (Falcon, Becton-Dickinson). Endothelial cells were detached with 120 IU/1 collagenase from Clostridium histolyticum (clostridopeptidase A. EC.3.4.24.3, Boehringer Mannheim) and 400,000 IU/1 hyaluronidase from ovine testis (hyaluronidate-4-glycanohydrolase, EC 3.2.1.35, Boehringer Mannheim) in BEM. The enzymatic mixture was injected into the vessels using a propylene-tipped syringe while the endothelial surfaces were gently rubbed together. The branches and ends of the vessels were not clamped. The specimens were placed in a 37°C incubator for 15 minutes. Then, arterial fragments were cut open longitudinally, and the detached cells were collected by washing the endothelial surfaces with protein-free BEM. Crude cell suspensions were pooled and washed twice with protein-free RPMI (Boehringer Mannheim) 100g, 10 minutes at 18°C). The final pellet was suspended in “complete RPMI 1640” containing 20% fetal calf serum, 1 g/l glucose, and sodium benzylpenicillin (500 IU/ml, final concentration). Freshly obtained passage 0 cells were seeded in 100-mm tissue culture Falcon petri dishes (approximately 10⁶ cells/10 ml complete RPMI 1640) and were allowed to remain undisturbed at 37°C in a humidified atmosphere with 5% CO₂ in air for 2–3 hours. As previously reported, cell attachment, confirmed by microscopic observations, was complete within this time.

The medium was removed, the attached cells were rinsed once with RPMI 1640 to remove the remaining red cells and debris, and EC culture preparations were done as follows: 1) For investigating acute drug effects, control confluent passage 0 ECs were prepared by covering freshly adhering, cleaned ECs with fresh complete RPMI 1640. The medium on new culture was changed each day for the first 2 days and subsequently once every 3 days. Under these conditions, passage 0 ECs reached confluency within 5–6 days of culture, showing the typical appearance of endothelium in a confluent monolayer (see Figure 1), i.e., a polygonal or cuboidal shape with large round nucleus and a cobblestone-like array. 2) For long-term exposure of passage 0 endothelial cells to organic nitrates, the freshly adhering cells were washed twice with RPMI 1640 and covered with 9 ml fresh complete RPMI 1640. ISDN, 2-ISMN, and 5-ISMN stock solutions were prepared immediately prior to use in ethanol (1 mg/ml) and were further diluted alone or as nitrate combinations in sterile, complete RPMI, giving the desired organic nitrate a final concentration one-tenth the original solution.
Isosorbide nitrates were a gift of Dr. Jospin, Theraplix Laboratories, Paris, France. The purity of the standards and ISN final concentrations were checked by ECD-GLC analysis as previously described. The addition of 1 ml of this intermediate dilution to the petri dishes provided the appropriate nitrate concentration in a 10-ml final volume; otherwise, the final volume was the same as that of the control culture.

When the cells reached confluency, they were promptly harvested as follows: The medium was removed, and endothelial cell monolayers were exposed to 0.02% EDTA (wt/vol)-trypsin (0.025%, wt/vol, BD-Meyrieux, 69260, Charbonnières, France) in calcium-magnesium free phosphate-buffered saline (PBS: KCl 0.026 M, KH₂PO₄ 0.014 M, NaCl 0.136 M, and Na₂HPO₄ 0.06 M, pH 7.4) until the cells detached from the bottom of the petri dishes. The resulting cell suspension was centrifuged at 100g for 10 minutes at 18°C. To remove EDTA, the cells were rinsed twice in RPMI 1640, in the same way each time, and resuspended in complete RPMI. These freshly confluent passage 0 endothelial cells were used after dilution to 5 x 10⁵ cell/ml in complete RPMI suspension, unless otherwise stated.

Aggregation Studies
Platelet aggregability was assayed in the absence of ECs, thus defining basal platelet aggregability, while in the presence of ECs, the mixed platelet-cell aggregability mirrored the ATECD. To characterize the inhibition of platelet aggregation by ECs, aggregation studies were performed by a modification of the turbidimetric method proposed by Born and modified by Curwen et al., using a Kontron Uvikon 860 double-beam UV-visible spectrophotometer connected to a PSY 80 Kontron computer (Roche-Kontron AG, Zürich, Switzerland), giving the percent transmittance in the time-drive mode. The wavelength used was 540 nm with a 4-nm spectral bandwidth. A sampling rate of 60 measurements/min eliminated most of the background noise encountered using the usual double-channel aggregometer (Figure 2). A siliconized cuvette with a siliconized stirring bar (1,100 rpm) in the test beam of the spectrophotometer thermostatted at 37°C was used. ECs in 0.1 ml RPMI (5 x 10⁵ cells/ml, unless otherwise specified) were added to 0.5 ml PRP (300 x 10⁶ platelets/l) in the cuvette. Then PBS (0.1 ml) was added either alone in drug chronic effect studies or with the drug to be tested at the appropriate
dilution in drug acute effect studies. The minimum and maximum transmittance limits of the display range were adjusted to give 0 and 100% light transmission readings with PRP and PPP, respectively. In controls, the corresponding buffers and media were added. Assessing basal platelet aggregability, 0.1 ml complete RPMI without cells was added to PRP as described above, which did not induce change in basal platelet aggregability. The cell-platelet suspension was stirred for 5 minutes at 37°C before increased doses of ADP were added. The aggregometer was a PSY 80 computer-assisted Kontron Uvikon 860 double-beam UV-visible spectrophotometer operating in the percent transmittance, time-drive mode. Measurement wavelength = 540 nm; spectral bandwidth = 4 nm; sampling rate = 60 measurements/min.

**Statistical Analysis of Data**

Statistical analysis of data was performed by one-way and multiple analyses of variance (ANOVA and MANOVA, respectively), except in those instances where Spearman rank correlation coefficient and Mann-Whitney U test were used. The results were obtained for duplicates or triplicates in three sets of independent experiments. The results in the text and graphics are expressed as means ± SD of aggregation extent (percent of control platelet value, i.e., the control platelet aggregation extent served as reference parameter for the mixed platelet-cell aggregability). This was done because MANOVA showed that there were significant differences between control results from one set of experiments to another (p < 0.05), although the same degree of accuracy was obtained in test results. This occurred because of slight variations in control platelet response to ADP from one platelet preparation to another.

**Results**

The basic characteristics of the passage 0 EC inhibitory effects on platelet aggregation were defined by evaluating the influence of endothelial cells on the aggregating strength of the proaggregating agent, ADP, and then by determining the influence of platelet-cell coincubation time, as well as the EC number, on platelet aggregating response to ADP. With the defined experimental conditions for ATECD measurement, pharmacologic modulations of ATECD by isosorbide nitrates were investigated under either an acute nitrate effect (platelet + control EC + drug + ADP) or a chronic effect (platelet + 5-day nitrate-treated EC + ADP).

**Platelet Aggregating Strength of ADP in Presence of Endothelial Cells**

In these studies, adenosine diphosphate was used as the aggregating agent since previous results from our laboratory, as well as others, demonstrated that isosorbide nitrates are potent inhibiting agents of ADP-induced platelet aggregation but poor inhibitors of arachidonate-, epinephrine-, and collagen-induced aggregation. In addition, ADP is released by platelets during secondary aggregation and thus is a common intermediate to platelet aggregation induced by various compounds. Platelet aggregation in response to 15 × 10⁻⁶ M ADP resulted in a maximal aggregation in the absence of ECs (data not shown). To determine the influence of ECs on ADP aggregating strength, 5 × 10³ ECs in 0.1 ml RPMI were incubated with 0.1 ml PBS + 0.5 ml PRP (1.5 × 10⁸ platelets) in the aggregometer at 37°C for 5 minutes, using doses of 15–150 × 10⁻⁶ M ADP as the aggregating agent. The results displayed in Figure 2 show that 150 × 10⁻⁶ M ADP resulted in maximal platelet aggregation and was equivalent to the control platelet aggregation. A higher ADP concentration did not increase the extent of platelet aggregation, even in the presence of larger amounts of cells (data not shown). The concentration of ADP that resulted in maximal platelet aggregation in the presence of ECs (150 × 10⁻⁶ M) was used in all subsequent phases of the experiment.

**Kinetic Characteristics of Endothelial-Cell Inhibitory Effects on Platelet Aggregation**

The kinetics of EC-induced inhibitory effect on ADP-induced platelet aggregation were determined by...
incubating $5 \times 10^4$ ECs with platelets under the previously described experimental conditions, using increasing intervals of time (1–10 minutes) prior to the addition of $150 \times 10^{-6}$ M ADP. The results are shown in Figure 3. It is apparent that, due to the presence of ECs, both the extent of platelet aggregation and the rate at which the aggregation plateau is reached (i.e., the aggregation velocity) is time-dependent and requires a 5-minute coincubation. The aggregation extent remained constant for the next 5 minutes while the aggregation velocity continued to decrease.

These results demonstrate that a 5-minute coincubation for ECs and platelets is adequate to evaluate the extent of ADP-induced platelet aggregation in the presence of cells.

Influence of Endothelial Cells on ADP-Induced Platelet Aggregation

To investigate the antiplatelet activity of freshly prepared confluent passage 0 endothelial cells, increasing quantities of cells ($10^3$–$10^6$ cells) were incubated with $1.5 \times 10^8$ platelets for 5 minutes prior to aggregation as described above. As shown in Figure 4, the presence of $5 \times 10^3$ ECs failed to induce significant change in $150 \times 10^{-6}$ M ADP-induced aggregation of $1.5 \times 10^6$ platelets. By increasing the number of cells in the aggregation suspension, platelet aggregation was progressively inhibited. The inhibition curve was sigmoid: A 50% inhibition of platelet aggregation required an average of $2 \times 10^4$ cells, and $5 \times 10^5$ cells resulted in a full inhibition of platelet aggregation. The presence of ECs, therefore, greatly affected the extent of platelet aggregation. Shape changes of aggregation curves were apparent when more than $10^4$ cells were added to the cuvette. A concentration of $3 \times 10^8$ platelets/ml was routinely used since lowering platelet density was found to result in narrowing changes of light transmission in response to ADP. Moreover, the use of higher platelet density would have required freshly prepared PRP to be concentrated. Finally, similar platelet density was used as in routine basal platelet aggregability.

Influence of Endothelial Cells on ISN Effects Against Platelet Aggregation

The antiplatelet activities of ISN were investigated in the presence of freshly prepared, confluent passage 0 endothelial cells by incubating $1.5 \times 10^8$ platelets with increasing ISN concentrations ($10^{-5}$ M, $5 \times 10^{-5}$ M, and $10^{-4}$ M) in the presence of varying amounts of cells from $10^3$ to $5 \times 10^4$. Higher cell quantities were not investigated because they resulted in spontaneous platelet inhibition greater than 90% of control platelet aggregation, thus corresponding to excessive back-

![Figure 3](http://circres.ahajournals.org/)

**Figure 3.** Kinetic analysis of inhibitory effects of endothelial cells (EC) on platelet aggregation. ECs ($5 \times 10^4$) were incubated with platelets as described in Figure 2 but for increasing intervals of time (1–10 minutes) prior to the addition of $150 \times 10^{-6}$ M ADP. Aggregation was recorded as in Figure 2.
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FIGURE 4. Influence of endothelial cell number on ADP-induced platelet aggregation. Increasing quantities of cells were added to $1.5 \times 10^9$ platelets prior to aggregation by $150 \times 10^{-6}$ M ADP, as described in Figure 2. Results are expressed as percent (mean ± SD) of control platelet aggregation from 3 experiments.

Figure 5. Influence of increasing numbers of ECs on platelet aggregation with various ISDN concentrations under acute pharmacologic conditions. Platelets ($1.5 \times 10^9$) were incubated according to procedure described in Figure 2.

ISDN doses of $5 \times 10^{-5}$ M and above were efficient even in the presence of a few ECs, while higher concentrations of ISDN ($10^{-3}$ M) required more than $5 \times 10^3$ cells to inhibit further platelet aggregation. It is, therefore, apparent that ISDN antiplatelet properties are dependent on both ISDN concentration and EC number. This multiple-variable dose-response relation is demonstrated by the parallelism between ISDN concentration-dependent dose-response curves and the aggregation extent vs. number of cells (Figure 5).

In the absence of ECs, we previously reported that 2-ISMN was more potent than both ISDN and 5-ISMN against ADP-induced platelet aggregation (see Figure 5 vs. Figure 6). After evaluation of the influence of ECs on ISDN antiaggregating properties, those of the mononitrate metabolites were investigated under the same experimental conditions to determine if ECs influence the relative potencies of isosorbide nitrates in the same way. From the results shown in Figure 6, both 2-ISMN and 5-ISMN were in contrast to ISDN because both failed to elicit any EC-induced stimulatory changes in intrinsic (direct) antiplatelet properties. Moreover, the 2-ISMN direct antiplatelet effects, i.e., in the absence of cells, were found to be cumulative with intrinsic $10^4$ cells ATECD. Contrary to the 2-ISMN effects, no cumulative antiplatelet effects were observed between 5-ISMN and ATECD since there were no significant differences in ATECD whether incubated with or without 5-ISMN. These results clearly demonstrate that ISDN antiplatelet effects are influenced by the presence of endothelial cells, but this is not the case for its mononitrate metabolites. It is likely that addressing the antiplatelet properties of a drug in the presence of ECs mirrors the pathophysiologic situation more closely than measuring the direct antiplatelet effect of the drug alone. By comparing the cumulative antiplatelet properties of each ISN (direct
Influence of Endothelial Cells' Long-term Exposure to ISN on ATECD

By showing that ISDN can stimulate ATECD, our results demonstrate the existence of an endothelium-dependent ISN antiplatelet activity, which has been detected as an acute antiplatelet effect in the series of experiments described. However, this does not allow us to estimate the extent of such an effect under chronic exposure to ISDN. This situation reflects the pharmacologic situation encountered under therapeutic circumstances. The effects of ECs' long-term exposure to nitrates were investigated by cultivating freshly prepared confluent ECs in the presence of ISN alone or in combinations (see “Materials and Methods” section and Figure 7 legend). The results refer to acquired changes in ATECD since in this set of experiments, aggregation studies were performed in the absence of drug. By examination of the priming effect on ATECD by $10^{-3}$ M and $10^{-4}$ M ISDN, 2-ISMN, and 5-ISMN, it appeared that 1) ISDN and 2-ISMN were capable of magnifying ATECD, in the same manner ($p < 0.001$), while 5-ISMN was a much less potent agent; 2) 2-ISMN had a cumulating effect on ATECD with ISDN, while 5-ISMN overshadowed ISDN on ATECD but did not affect the other mononitrate, 2-ISMN; and 3) when ISDN was tested in combination with its two mononitrate metabolites, the overall priming effect of isosorbide nitrate on ATECD was less than 50% of that expected by cumulating individual ISN effects.

These results strongly support the following conclusions: 1) ISDN is capable of eliciting an ATECD-stimulation priming effect on ECs in culture; 2) 2-ISMN contrasts its absence of EC-mediated acute inhibition effect on platelet activity by showing a priming effect on ECs in a manner similar to ISDN; and 3) 5-ISMN is a poor antiplatelet agent. Moreover, 5-ISMN is capable of counteracting the priming effect of ISDN on ATECD.

Discussion

In these studies, we addressed the pharmacologic regulation of the properties of endothelial cells by isosorbide nitrate (ISN), which enables these cells to present a nonthrombogenic surface to the blood stream. The results presented here are in agreement with previous reports from several laboratories indicating that cultured animal ECs remain thromboresistant in vitro under well-controlled conditions. The term antithrombogenic endothelial cell defense (ATECD) was coined to refer to the overall properties enabling the endothelium to prevent circulating blood platelet aggregation adherence to the vascular wall.

Prior to pharmacologic investigations on ISN properties, we defined the conditions permitting studies on platelet behavior during platelet aggregation in an experimental model based on mixed-platelet–EC suspensions in human plasma. The aggregating agent was adenosine diphosphate (ADP) because we had previously found it to be the most sensitive agent to ISN platelet-inhibiting properties. There is no evidence that EC-induced inhibition of ADP-induced platelet aggregation is the major regulator of thrombosis in vivo. However, recent evidence suggests that ADP is involved in activating platelets in vivo. By continuous enzymatic removal of ADP and ATP from the blood plasma, it has been shown that the presence of ADP enhances the activation and thrombotic aggregation of arriving platelets. The minimal concentration of ADP that resulted in maximal platelet aggregation in EC presence ($150 \times 10^{-8}$ M) was tenfold that required for the same efficiency in EC absence. This is likely due to ADP hydrolysis by an endothelial ecto-ADPase and/or to uptake by endothelial cells, which contain purine receptors and have proven to be implicated in the removal of nucleotide from the blood.

The present studies indicate that isosorbide dinitrate (ISDN) is capable of inhibiting platelet activity in an endothelium-dependent manner that is not to be confused with antiplatelet direct effects of ISDN. Stimulation of the antithrombogenic properties of ECs with ISDN was dose-dependent for a constant number of cells. The specificity of this ISDN effect is demonstrated by the failure of its mononitrate metabolites to elicit any antiplatelet response from ECs since under these circumstances platelet aggregation did not respond to isosorbide mononitrites (ISMN). The mechanism of the EC-mediated antiplatelet ISDN effect remains to be elucidated. Under acute pharmacologic circum-
stances, in the presence of the drug, one has to consider that either EC participate in platelet inhibition by providing one or several molecules that combine with ISDN and further inhibits platelet aggregation, or that EC-dependent ISDN acute antplatelet properties result from a two-step process in which ECs are sensitized by ISDN prior to enhancing their naturally occurring antithrombogenic properties. Both hypotheses are currently under investigation in our laboratory. On the other hand, long-term exposure of ECs to ISN was found to result in increased ATECD-acquired changes. Under these conditions, 2-ISMN was as active as ISDN, while 5-ISMN was capable of counteracting ISDN effects. Under chronic conditions, the involvement of ISN is to be ruled out since aggregation studies were performed in the absence of the drug, and ISN should not be released from ECs because it does not accumulate in ECs during cell culture (P.H. Rolland et al., unpublished observation). By studying a reduced thiol may potentiate inhibition of platelet aggregation by nicotinic oxide-containing vasodilators, it has been shown that S-nitrosothiols inhibit platelet aggregation and are formed when platelets are incubated with nitrates. Therefore, it is tempting to consider that ECs may release a thiol-containing compound, reduced glutathione for instance, which may combine with ISDN to form S-nitrosothiols to inhibit platelet aggregation. This proposed mechanism for the immediate ISDN antplatelet effects in the presence of ECs would also explain the ISDN specificity regarding mononitrites since ISDN form S-nitrosothiols more easily than 2-ISMN and 5-ISMN, ISDN being approximately 500 times more reactant with sulfhydryl compounds to form nitrosothiols than its two mononitrite metabolites. These observations argue for a two-step mechanism for increasing the antplatelet properties in EC presence with ISDN.

The findings of this long-term exposure to a drug under pharmacologic conditions may amplify ATECD addresses the mechanisms responsible for endothelial cell antithrombogenicity. In our experimental model, bovine ECs and human platelets were incubated with autologous human plasma. This situation implies that ECs may interact with plasma coagulation proteins and platelets, which are possible targets for EC antiaggregating properties. There are excellent recent reviews on the antithrombogenicity of ECs and pharmacologic modulations of thrombosis. At present, the mechanisms responsible for compatibility of ECs with blood are not completely understood, although several hypotheses have attempted to explain the thromboreisresistant character of intact vascular wall endothelial lining. These include an electrostatic repulsion of blood cells by ECs, synthesis of plasminogen activator, release of an ecto-ADPase, and production of heparin sulfate. In addition, the vascular endothelium synthesizes prostacyclin, a potent antiaggregatory substance with vasodilator properties (for review, see Moncada44). Prostacyclin has been implicated in the thromboresistance of intact endothelium, but other reports cast doubt on this conclusion. The basis for ATECD remains to be determined, especially the precise mechanism(s) by which ISNs are used as stimulators.

Finally, the existence of an endothelium-dependent antplatelet effect of ISDN strongly suggests that ISDN owes its in vivo effects to the direct antplatelet properties of both ISDN and its mononitrate metabolites on the one hand, and the EC-dependent antplatelet ISDN properties on the other hand. The influence of ISN in various combinations on ATECD should therefore be taken into account when the biologic relevance of the effect of ISDN is examined, although the precise contributions of 5-ISMN and 2-ISMN to the effects of ISDN, especially to its duration of action, remain to be clarified.

The present results must be considered cautiously before conclusions to the situation in human beings are drawn. However, it is still possible that 5-ISMN, overshadowing ISDN and 2-ISMN antplatelet properties, would explain why ISDN and perhaps other nitrates have not proven efficient protection against myocardial infarction. The classic view linking pathogenesis of myocardial infarction to thrombotic diseases is now substantiated by recent findings that highlight the need for efficient antithrombotic therapeutic agents. However, the anticoagulant and antiplatelet therapeutic use have provided marginal benefit in prevention of tissular ischemia and infarction. By showing that the natural antithrombogenic endothelial cell defense may be pharmacologically modulated and stimulated, it is perhaps not presumptuous to consider that a new therapeutic target (i.e., the prevention of aggregating platelets and/or platelet aggregates adherence to, and/or aggregation on, the vascular wall) should now be investigated clinically.

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