Effect of Sulfipyrazone on Homocysteine-Induced Endothelial Injury and Arteriosclerosis in Baboons

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SUMMARY. The effect of sulfipyrazone on endothelial injury induced by homocysteine has been studied both in vitro, using cultured human umbilical vein endothelial cells, and in vivo, using a primate model of homocysteine-induced arteriosclerosis. Oral sulfipyrazone (250 μmol/kg body weight per day in three divided doses) in eight chronically homocystinemic baboons (0.14 ± 0.04 mM plasma homocystine) decreased the extent of aortic endothelial injury as measured morphometrically by silver staining techniques, compared with six untreated comparably homocystinemic animals (denuded surface averaged 0.5% with range 0–2.1 vs. 7.7 ± 1.6%, respectively; P < 0.001). Sulfipyrazone therapy to homocystinemic baboons also normalized platelet survival and turnover measurements (5.1 ± 0.4 days and 70,000 ± 11,000 platelets/μl per day vs. 2.8 ± 0.6 days and 179,000 ± 19,000 platelets/μl per day in untreated homocystinemic controls; P < 0.001). Sulfipyrazone therapy also reduced the size and frequency of homocysteine-induced intimal lesion formation (P < 0.001). Although sulfipyrazone reduced the amount of specific 51Cr release from cultured human umbilical vein endothelial cells induced by 10 mM homocysteine after 24 hours of co-incubation, no effect was observed in assays of endothelial cell detachment when sulfipyrazone (10^-5 M) or its thioether metabolite were pre- or co-incubated during 24 hours with homocysteine (2.5–10 μM). These data suggest that sulfipyrazone may protect endothelial cells from injury in vivo by some apparently indirect mechanism. (Circ Res 53: 731-739, 1983)

ARTERIOSCLEROTIC lesions may be produced experimentally by inducing endothelial denudation by a variety of methods, including mechanical removal (Bjorkerud and Bondjers, 1971; Stemerman and Ross, 1972), immune injury (Minick and Murphy, 1973; Hardin et al., 1973), and chemical cytotoxicity (Harker et al., 1974, 1976). In normocholesterolemic primates, maximal lesion formation occurs within 3 months after mechanical injury with a balloon catheter, and the lesions regress during the subsequent 3 months as the overlying endothelium regenerates (Stemerman and Ross, 1972). Additional studies support the concept that this process is platelet dependent, as shown by the observations that intimal proliferative lesions produced by chronic indwelling arterial catheters or balloon catheter deendothelialization can be prevented by severe thrombocytopenia (Moore et al., 1976, Friedman et al., 1977). In addition, normocholesterolemic or hypercholesterolemic swine homozygous for von Willebrand's disease, a genetic defect of decreased platelet-surface interactions, show a reduced frequency of atherosclerotic lesions compared with age- and sex-matched control normocholesterolemic or hypercholesterolemic swine (Fuster et al., 1978). These observations suggest that injury to the endothelium is important in the initiation of at least some types of arteriosclerotic lesions, and that impairment in platelet-subendothelial interactions may prevent lesion formation (Ross and Glomset, 1976).

Chronic homocystinemia produced in primates by continuous intravenous homocysteine infusion results in patchy endothelial cell injury and focal proliferative intimal smooth muscle cell lesions similar in appearance to early atherosclerotic lesions in man. Homocystinemic animals treated with dipyridamole, an inhibitor of platelet function, show a marked reduction in intimal lesion formation without altering the amount of injured endothelium (Harker et al., 1976). Sulfipyrazone is a commonly used platelet function inhibitor whose mechanism of action is incompletely understood. Sulfipyrazone has been reported to normalize platelet survival time and platelet turnover in patients with various arterial thromboembolic disorders (Smythe et al., 1965), cardiac valvular disease (Weily and Genton, 1970), and coronary artery disease (Steele et al., 1975). On the basis of controlled clinical trials, sulfipyrazone has also been reported to reduce the frequency of thrombotic occlusion of arteriovenous cannulas in patients on chronic hemodialysis (Kaegi et al., 1974), to reduce cardiovascular mortality in elderly male patients with prior history of stroke (Blakely and Gent, 1975), and to reduce sudden death and thrombotic events among patients with recent myocardial in-
farction (The Anturane Reinfarction Trial Group, 1978; Report from the Anturane Reinfarction Italian Study, 1982). However, ex vivo platelet function studies in patients taking the usual therapeutic doses of sulfinpyrazone are minimally affected. The bleeding time is usually normal, and platelet aggregation to most agonists is not inhibited (Packham and Mustard, 1977). Sulfinpyrazone is only a weak inhibitor of platelet cyclooxygenase, compared with acetylsalicylic acid (Ali and McDonald, 1978), and has only a modest effect on platelet adherence (Baumgartner et al., 1976; Cazenave et al., 1975).

The capacity of sulfinpyrazone to prolong platelet survival in vivo without notably affecting platelet function in vitro has led to the suggestion that sulfinpyrazone may act primarily to prevent or modify endothelial injury.

In the present study, we have examined the effects of sulfinpyrazone on homocysteine-induced endothelial injury in vitro, using cultured human umbilical vein endothelial cells, and in vivo, using a baboon model of homocysteine-induced endotelial denudation and arteriosclerosis.

Methods

Cell Isolation and Identification

Endothelial cells were obtained by collagenase (Calbiochem, B grade) treatment of human umbilical veins, as previously described (Wall et al., 1980). Cells were pooled from at least three cords. The cells were grown to a confluent monolayer in Waymouth's medium MB 752/1 (Irvine Scientific) enriched with 20% pooled human serum (PHS). The demonstration of cell-associated factor VIII antigen by fluoresceinated rabbit antihuman factor VIII antibody and Weibel-Palade bodies by transmission electron microscopy (Weibel and Palade, 1964) confirmed the identity of the cells. Only primary passage cells were used for these experiments. Whole blood was collected from 50-60 normal donors and allowed to clot overnight at 4°C (obtained through the courtesy of the Puget Sound Blood Center), and the serum was collected and pooled. The resulting serum pool was filtered through a 0.2-μm Millipore filter, heat inactivated at 56°C for 30 minutes, and stored at -70°C until needed.

Radiochromium Release Assay

Cells were harvested with 0.05% trypsin and replated in multwell plates (Falcon 3008) at a density of 100,000 cells/well (Wall et al., 1980). At this density, the endothelial cells formed a confluent monolayer. The cells were allowed 24 hours to attach and were then labeled with 51Cr activity (5.0 ml medium containing 25 μCi of 51Cr; New England Nuclear) for 90 minutes. Excess label was removed by three washes with phosphate-buffered saline containing calcium chloride (1 mM) and magnesium chloride (0.5 mM), and a fourth wash with equal parts of Waymouth's medium and PBS, in a 30-minute incubation. Test material then was added in serum-free Waymouth's medium to the labeled cells for periods of 4 and 24 hours. Percent radiochromium released was calculated by dividing 51Cr activity released during test incubation by maximal releasable 51Cr activity. Maximum release was determined by incubation with 1% Triton X-100 (New England Nuclear) and was generally about 20,000 counts/min, or ~90% of the radiolabel from the cell layer. The spontaneous release in control wells after a 4-hour incubation was less than 15%, but after 24 hours of incubation, was often 50% of the maximum release.

Cell Detachment Assay

Cell detachment was determined by direct cell counting, as previously described (Wall et al., 1980). Briefly, primary passage endothelial cells were harvested with 0.05% trypsin and 0.02% EDTA in normal saline (Gibco) and plated in 20% PHS/Waymouth's medium in microtiter plates (Falcon 3040) at a density of 10⁵ cells/cm². Cells were incubated for 24–48 hours to form a confluent monolayer. Test medium then was added for an additional 24-hour incubation. After incubation, the cell layers were washed five times with 0.5% bovine serum albumin (BSA) (Miles Laboratories) in Dulbecco's phosphate-buffered saline (PBS) (Gibco) using rapid pipetting and vacuum aspiration (5 pounds per square inch). Following the washing, all of the residual adherent cells excluded trypan blue. Cell number was determined directly by electronic counting (Coulter model B, Coulter Electronics) after harvesting attached cells with 0.05% trypsin and 0.02% EDTA in normal saline. Results were expressed as:

\[
\text{% Detachment} = \frac{\text{control} - \text{test}}{\text{control}} \times 100%.
\]

Reagents

dl-Homocysteine thiolactone (Aldrich Chemical) was dissolved as a 10⁻¹ M stock in PBS immediately before final dilution in 20% PHS/Waymouth's medium. Sulfinpyrazone and its thioether metabolite (gifts from CIBA-Geigy) were dissolved in 0.05 N NaOH as a 10⁻³ M stock immediately before final dilution in 20% PHS/Waymouth's medium. In all experiments, sulfinpyrazone and its thioether analog were compared with a similarly diluted NaOH solvent control.

Baboon Homocysteine Vascular Model

Seventeen male baboons (Papio cynocephalus) were studied, three nonhomocystinemic normal control animals, six untreated chronic homocysteine infusion control animals, and eight animals receiving both chronic homocysteine infusions and oral sulfinpyrazone. Two normal animals not receiving homocysteine and three homocystinemic animals not receiving treatment were studied concurrently with the first set of four sulfinpyrazone-treated homocysteinemic animals. The third normal control animal and the three remaining untreated homocystinemic control animals were studied after the examination of the second set of four treated homocysteinemic animals. The animals, which weighed 10–12 kg, had hematocrits of 40 ± 3%, leukocyte counts of 5,000/μl ± 1,200, and platelet counts of 279,000/μl ± 59,000. They were free of tuberculosis, dewormed, and observed to be disease-free for at least 6 weeks before use. To facilitate continuous intravenous infusion and repeated blood sampling, a femoral arteriovenous (AV) Silastic shunt was used, 0.24 cm in internal diameter and 25 cm long, with Dacron sewing cuffs at skin exit sites. Platelet survival was not measurably shortened by this cannula alone (Harker and Hanson, 1979).

A solution of dl-homocysteine (0.3 g/kg body weight, Aldrich Chemical Co., Inc.) or a control solution alone...
The bleeding time was measured by the standardized template technique (Harker and Slichter, 1972); the mean bead retention test and aggregation induced by ADP (0.5, by its average time in circulation. In 24 normal animals, labeled from the concentration of plasma fibrinogen divided by the natural logarithm of 2, averaged 2.6 ± 0.2 in 35 normal animals. Fibrinogen turnover, from the half-time distribution survival time in days, and corrected for recovery. In 35 normal male baboons, platelet survival was 5.5 ± 0.3 days. Platelet and fibrinogen concentration, survival, and turnover were measured at monthly intervals during the infusion period. Plasma cholesterol and triglyceride levels were obtained prior to the infusion studies and before sacrifice.

Sulfinpyrazone, a gift from CIBA-Geigy, was given orally to eight animals throughout the 3-month period of homocysteine infusion in dosages of 250 μmol/kg per day (100 mg/kg per day) in three divided doses. Plasma sulfinpyrazone levels were also measured serially following oral ingestion of the drug on two different occasions before the animals were killed. Determinations were carried out by CIBA-Geigy Biopharmaceutical Research Center, Cedex, France, by means of liquid chromatography (Lecollion and Souppart, 1976).

Platelet counts were measured with an electronic particle counter on peripheral blood collected in EDTA by a previously described method (Harker and Finch, 1969). The mean platelet count of 50 normal baboons was 279,000/μl ± 59,000 (± 1 sd). Platelet survival was determined from the disappearance of radioactivity from blood sampled 8 to 10 times after the injection of autologous 51Cr-labeled platelets (Harker and Hanson, 1979). Three-milliliter samples of whole blood, collected in EDTA twice daily for 3 days and daily for 3 additional days, were lysed with 0.1 ml of SDS and counted for radioactivity in a γ spectrometer. Platelet survival time was determined by computer least squares fitting to γ functions (Harker and Hanson, 1979). The proportion of labeled platelets remaining within the systemic circulation following infusion (i.e., "recovery") was calculated from the platelet activity per milliliter extrapolated to zero time, multiplied by the estimated blood volume, and divided by the platelet 51Cr activity injected. Platelet consumption, measured as platelet turnover per microliter of blood per day, was calculated from the peripheral platelet count divided by the platelet 51Cr activity injected. Platelet consumption, measured as platelet turnover per microliter of blood per day, was calculated from the peripheral platelet count divided by the platelet survival time in days, corrected for recovery. In 35 normal male baboons, platelet survival was 5.5 ± 0.3 days. Platelet recovery averaged 85 ± 5% and platelet turnover was 59,000 ± 7000 platelets/μl per day.

For calculating fibrinogen turnover, the concentration of fibrinogen was estimated by a spectrophotometric method (Jacobsson, 1955) and was 2.71 ± 0.22 mg/ml in 24 normal animals. Labeling of normal fibrinogen with 125I was performed by the method of Takeda (1966). Fibrinogen survival, determined from the half-time disappearance divided by the natural logarithm of 2, averaged 2.6 ± 0.2 in 35 normal animals. Fibrinogen turnover, used as a measure of fibrinogen consumption, was calculated from the concentration of plasma fibrinogen divided by its average time in circulation. In 24 normal animals, fibrinogen turnover was 1.00 ± 0.10 mg/ml per day. Platelet function was measured by bleeding time, glass-bead retention test and aggregation induced by ADP (0.5, 1.0, 1.5, 2.0, and 10.0 μM final concentration), collagen (1, 5, and 20 μg/ml), and epinephrine (1, 2.5, and 10 μg/ml). The bleeding time was measured by the standardized template technique (Harker and Slichter, 1972); the mean value in 24 normal baboons was 4.0 ± 1.2 minutes. Platelet aggregation was estimated from changes in optical transmission of 0.012 m sodium-citrate plasma at 37°C with a concentration of 300,000 platelets/ml (Born and Cross, 1963). Plastic equipment was used throughout, and platelet-rich plasma, freshly drawn, was kept capped at room temperature during the 30–70 minutes before testing. Platelet retention by glass-bead columns was measured by the Bowie modification of the Salzman method (Bowie et al., 1969), blood being drawn by two-syringe technique (plastic) without anticoagulant and immediately passed through the standard column of beads by a constant infusion pump at the rate of 2 ml/min. The delay between blood drawing and initiation of flow through the beads was less than 10 seconds.

Homocystine was measured courtesy of Dr. C. Ronald Scott, University of Washington, Seattle, using column chromatography according to a modification of the procedure of Sparkman et al. (1958).

Morphological Procedures

Three types of vessel preparations were obtained: (1) lower abdominal aorta, iliac, and femoral arteries for light and electron microscopic examination of lesion formation, (2) carotid arteries for [3H]thymidine incorporation by endothelial cells, and (3) thoracic and upper abdominal aorta for silver stain quantification of endothelial coverage. Specimens were obtained under general (halothane) anesthesia and assisted respiration in the following way. After opening the abdomen, we carefully freed the aorta and inferior vena cava by sharp dissection, without affecting flow. The inferior vena cava then was cannulated to establish isolated venous drainage from the lower limbs, and immediately thereafter the abdominal aorta was cannulated, proximally flushed free of blood elements with 5% glucose solution at 100 mm Hg of hydrostatic pressure, and perfused with buffered half-strength Karnovsky's fixative under 100 mm Hg of pressure for 20 minutes in vivo (Harker et al., 1976; Haudenschild et al., 1972). Once perfusion-fixation of the distal arteries was underway, the carotid arteries were freed by sharp dissection, isolated, and carefully removed. The artery segments were gently flushed free of blood with saline and excess adventitia removed for [3H]thymidine studies. Immediately after the carotid arteries had been removed, the chest was entered, the arch of the aorta quickly cannulated, and drainage outflow was established above the renal arteries. The aorta then was cleared of blood, with 5% glucose solution at 100 mm Hg pressure. In vivo staining then was carried out by pressure perfusion of the thoracic and upper abdominal aorta with 0.3% silver nitrate solution at a pressure of 100 mm Hg for 20 minutes (Poole et al., 1958). After perfusion-fixation, the vessels were removed by sharp dissection. The foregoing procedure ensured the procurement of three different artery preparations from a single living animal while using isolated regional pressure-perfusion fixation.

Intimal Lesion Score (ILS)

At the time of sacrifice, the aorta was cannulated below the renal vessels, flushed free of blood with 5% glucose solution, and then perfused with buffered half-strength Karnovsky's fixative under 100 mm Hg of hydrostatic pressure for 20 minutes in vivo. Vessels were subsequently removed by sharp dissection and placed in half-strength Karnovsky's fixative for 6 hours at 4°C. Segments
from abdominal aorta and each iliac artery were removed for scanning EM. The remaining vascular tissues were subsequently cut into approximately 1-mm rings and returned to the fixative for another hour at 4°C. They were then washed in 0.1 M cacodylate buffer containing 0.2 M sucrose (pH 7.3) and subsequently postfixed in 1% osmium tetroxide buffered with s-collidine or with phosphate buffer (pH 7.3) for 1-1/2 hours, followed by enblock staining with 2% uranyl acetate for 1/2 hour. After having been embedded in epoxy resin, thick sections were cut at 1 μm as circles and stained with a combination of basic fuchsin and azure II-methylene blue for light microscopy (Huber et al., 1968). Selected thin sections were cut at a thickness of approximately 800 Å, for transmission electron microscopy. These sections were stained with lead citrate, followed by uranyl acetate. All of the sections for electron microscopy were examined in an AEI 801 electron microscope.

Five random sections were prepared from comparable sites of each of five vessels (abdominal aorta, right, and left iliac arteries, right and left femoral arteries) for light microscopic scoring of intimal lesions. The lesions were graded by two observers in a "blind" manner according to the cell layer depth of intimal smooth muscle cells: grade 0, only occasional intimal smooth muscle cells; grade 1, 1-5 cells deep; grade 2, 6-10 cells; grade 3, 11-15 cells; grade 4, 16-20 cells; grade 5, more than 20 cells deep. The overall score was calculated for each animal by summing the scores of all 25 sections examined (possible range of scores was 0-125).

[3H]Thymidine Labeling Index (TLI)

Both carotid arteries were removed by sharp dissection, flushed with saline and cut into 20-mm lengths. Whole segments were placed in 3-4 ml of medium TC 199 containing 1 μCi [3H]thymidine/ml and incubated at 37°C for 4 hours. The vessel segments were removed, trimmed, opened longitudinally, and mounted flat for fixing 24 hours in 10% neutral buffered formaldehyde. The endothelium was prepared for autoradiography by the method of Schwartz and Benditt (1973) which involved the following procedures. The luminal surface of the specimen was embedded onto a collodion slide, leaving the adventitial surface exposed. The adventitial surface was then embedded in gelatin and bonded to a gelatin-coated slide by fixation with formaldehyde while the specimen and the coated slide were held together under pressure. Subsequently, the collodion overlying the endothelial cells was removed with ether and alcohol, thereby exposing the luminal surface of endothelial cells. After coating with autoradiographic emulsion, the specimens were stored at 4°C for 2 weeks, then developed using Kodak NTB-2 emulsion. The labeled cells were scored in a blinded manner as labeled or unlabeled, and the [3H]thymidine labeling index (TLI) represented the ratio of these values expressed as a percentage.

Endothelial Cell Loss (ECL)

Pressure-perfusion fixation was carried out on the thoracic and upper abdominal aorta with 0.3% silver nitrate solution, at the time of sacrifice, in vivo (Poole et al., 1958; Haudenschild et al., 1972; Harker et al., 1976). The entire endovascular surface of whole-thoracic aorta mounts was then examined morphometrically in a blinded manner by means of a grid micrometer, and the proportion of the surface that was not covered by endothelial cells was determined. Although the endothelium was shown to be intact in normal control animals, perfusion with silver nitrate without prior glutaraldehyde perfusion overestimates the actual amount of denuded luminal surface present in vivo.

Statistical analysis was carried out through the PROPHET System developed by the Chemical Biological Information Handling Program of the Biotechnology branch of the Division of Research Resources, National Institutes of Health. Significance was determined by t-test comparisons of means, for the in vitro studies, and by the Wilcoxon Mann-Whitney rank sum test, for the animal studies (Zar, 1974).

Results

In Vivo Studies

Endothelial Cell Injury

Endothelial cell coverage was completely intact in three normal animals not given homocysteine when vessels were prepared by pressure-perfusion fixation in vivo, using either 0.3% AgNO3 staining for light microscopy or scanning electron microscopy (Table 1). These observations were consistent with the results of previous control studies (Harker et al., 1976). Patchy endothelial cell loss was observed in six untreated, concurrent, positive control homocystinemic animals. The overall loss of aortic endothelium in these untreated animals was 7.7 ± 1.6% of the aortic surface (P < 0.001 compared with normal control animals) and corresponded to a mean plasma homocystine concentration before sacrifice of 0.157 mM ± 0.026 (Table 1). A correlation was observed between endothelial cell loss and homocystine concentration (Table 1) with a coefficient of 0.801 (P < 0.01). These results in a limited number of homocystinemic control animals were essentially identical to those in a larger group of homocystinemic baboons in an earlier study (Harker et al., 1976).

In contrast, eight animals treated with oral sulfipyrazone showed a minimal loss of aortic endothelium (Table 1), averaging 0.5%, with a range of 0-2.1% (P < 0.001 compared with the six untreated homocystinemic animals), despite the mean plasma homocystine concentration of 0.128 ± 0.043 mM (Table 1). The basal plasma concentration of sulfipyrazone in these animals averaged 1.95 ± 1.69 μg/ml with a mean peak value 1 hour after drug ingestion of 24.2 ± 14.4 μg/ml. Administration of drug to treated animals was associated with a significant change in the relationship between homocystine concentration and endothelial cell denudation (P < 0.01), compared with untreated homocystinemic animals.
Platelet Consumption

Platelet survival in the six control untreated homocystinemic animals was reduced to 2.8 ± 0.6 days compared with 5.4 ± 0.3 in the normal control group and 5.3 ± 0.3 baseline before the induction of homocystinemia (P < 0.001; Table 1). Reciprocally, platelet turnover was increased 3-fold in the homocystinemic animals, i.e., 179,000 ± 19,000 compared with 63,000 ± 4,000 platelets/µl per day, baseline (P < 0.001). Sulfinpyrazone therapy normalized platelet survival (mean 5.1 ± 0.4 days, P > 0.05, compared with baseline measurements before homocystinemia; P < 0.01, compared with untreated homocystinemic controls). Platelet turnover was also normalized in the sulfinpyrazone-treated animals (70,000 ± 11,000 platelets/µl per day; P > 0.05, compared with baseline values).

In homocystinemic animals, fibrinogen survival and turnover were not changed significantly from control values, i.e., 2.4 ± 0.2 days and 1.2 ± 0.2 mg/ml per day, respectively, compared with 2.5 ± 0.2 days and 1.1 mg/kg per day in control animals. Fibrinogen kinetics were not altered by sulfinpyrazone treatment.

Platelet aggregation induced by ADP, epinephrine, and collagen was not significantly different in six sulfinpyrazone-treated animals, compared with six untreated homocystinemic animals or eight normal control animals (P > 0.2). Similarly, the bleeding time was not significantly prolonged by sulfinpyrazone therapy (4.8 ± 2.5 compared with 4.2 ± 2.0 minutes for six untreated homocystinemic animals and 4.0 ± 1.2 minutes in eight normal control animals, P > 0.1). Platelet retention by glass beads was minimally reduced in the sulfinpyrazone-treated animals (36 ± 11% compared with 43 ± 12% in homocystinemic control animals, P < 0.05).

Endothelial Cell Regeneration

In homocystinemic animals, fibrinogen survival and turnover were not changed significantly from control values, i.e., 2.4 ± 0.2 days and 1.2 ± 0.2 mg/ml per day, respectively, compared with 2.5 ± 0.2 days and 1.1 mg/kg per day in control animals. Fibrinogen kinetics were not altered by sulfinpyrazone treatment.

In control animals, the [3H]thymidine labeling index (TLI) of endothelial cells averaged 0.0011 ± 0.0003%. Animals with induced homocystinemia showed a greater than 25-fold increase in the incorporation of [3H]thymidine by endothelial cells, i.e., 0.0247 ± 0.0195%, P < 0.001. Although sulfinpyrazone-treated animals had a modest but significant increase in TLI compared with normal animals (0.0048 ± 0.0049%, P < 0.01), a significant decrease was also observed when compared with untreated homocystinemic animals (P < 0.01).

Table 1

<table>
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<tr>
<th>Animals</th>
<th>Homocystine concentration (mM)</th>
<th>Platelet survival (days)</th>
<th>[3H]-Thymidine labeling index (%)</th>
<th>Endothelial cell loss (%)</th>
<th>Intimal lesion score</th>
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* Variances are given as ± 1 SD.
Effect on Intimal Lesion Formation

For every animal studied, five random sections taken from comparable sites in each of five different arteries were graded 0-5 (see Methods). Single intimal smooth muscle cells were occasionally observed in control animals (scores averaged 4 ± 3, range 1–7).

The six homocystinemic positive control animals developed eccentric myointimal lesions with intimal lesion scores averaging 56 ± 9 (Table 1). The fine structure of the lesions was typical of early lesion formation similar to the fibromusculoelastic arteriosclerotic lesions in man (Harker et al., 1976).

The mean intimal lesion score of the eight animals receiving oral sulfinpyrazone throughout the 3-month period of homocystinemia was 14 ± 6. This result differed from the results in normal animals (4 ± 3; \( P < 0.01 \)) and from the animals receiving homocystine without drug treatment (56 ± 9; \( P < 0.01 \)). The endothelium was preserved in the sulfinpyrazone-treated animals, even at sites containing small lesions, despite the presence of homocystinemia (Fig. 1). Examples of such regions in sulfinpyrazone-treated animals can be seen in Figure 1. Junctional complexes and endothelial cell ultrastructure appeared unaltered from normal in the sulfinpyrazone-treated animals.

Serum cholesterol concentration for the control, homocystinemic, and sulfinpyrazone-treated homocystinemic groups were 99 ± 6, 106 ± 7, and 92 ± 5, respectively. No correlation was observed in these animals between cholesterol levels and either lesion scores or the presence of lipid within the lesions.

In Vitro Studies

Chromium Release Assay

Incubations of endothelial cells with homocysteine, 10 mM, for 4 hours produced minimal but significant chromium release (control, 13.4 ± 1.25%; homocysteine, 10 mM, 17.4 ± 0.9%). When the incubation was prolonged to 24 hours, the spontaneous release increased markedly (mean of 20 replicates averaged 52.2 ± 2.8%). With this high background, no injury was detectable at homocysteine concentrations of 1 and 5 mM. However, 10 mM homocysteine produced a significant increase in chromium release over control (20 replicates averaged 90.3 ± 3.2% with 10 mM homocysteine, compared with 52.5 ± 2.8 control; \( P < 0.001 \)). When sulfinpyrazone (10⁻⁵ M) was added to the 24-hour incubation mixture, there was a significant reduction in chromium release at the 10 mM homocysteine concentration (20 replicates gave means of 90.3 ± 3.2% with 10 mM homocysteine, 76.6 ± 1.6% with homocysteine 10 mM, plus sulfinpyrazone 10⁻⁵ M; \( P < 0.001 \)).

Cell Detachment Assay

Because this assay employs direct counts of adherent cells remaining after exposure to homocysteine and does not require a radiolabel, the high background in the chromium release assay seen at 24 hours is avoided, and a significant dose-dependent injury at homocysteine concentrations of 2.5 to 10.0 mM could be detected (Table 2). Incubation with sulfinpyrazone, 10⁻⁴ to 10⁻⁶ M, had no effect.

FIGURE 1. This low magnification electron micrograph was taken from a representative area of one of the largest lesions found in a sulfinpyrazone-treated homocystinemic baboon. The endothelial cells are intact. Several intimal smooth muscle cells can be seen in the slightly thickened intima. Each smooth muscle cell is surrounded by a relatively large amount of newly formed connective tissue. 4400×
on endothelial cell detachment induced by 10 mM homocysteine (Table 2). In an attempt to see whether a protective effect of sulfinpyrazone could be demonstrated at lower homocysteine concentrations or with pre-incubation of the endothelial cells with sulfinpyrazone, we incubated endothelial cells with sulfinpyrazone for 18 hours before, and then for 24 hours during, exposure to homocysteine. This 48-hour incubation with sulfinpyrazone (10⁻⁴ M) itself produced endothelial cell detachment (15.8 ± 2.7% detachment, mean of eight replicates); however, sulfinpyrazone (5 × 10⁻⁵ M) produced no detectable endothelial cell detachment during the same period of co-incubation. This concentration of sulfinpyrazone failed to prevent detachment induced by 2.5 and 5.0 mM concentrations of homocysteine (Table 2). The thioether metabolite of sulfinpyrazone (Dieterle et al., 1975) was also ineffective in preventing homocysteine-induced endothelial detachment (Table 2).

**Discussion**

The results of the animal studies demonstrate that sulfinpyrazone in plasma concentrations that approximate levels observed clinically (Dieterle et al., 1975) reduces both the amount of endothelium lost with a given plasma concentration of homocysteine and the rate of endothelial regeneration (Table 1). Sulfinpyrazone therapy also normalizes platelet survival time and turnover in homocystinemic baboons. Moreover, there is a marked reduction in the smooth muscle proliferative lesions of homocysteine-induced arteriosclerosis in animals treated with sulfinpyrazone. These observations may document a potentially useful effect of sulfinpyrazone in the prevention of acute and chronic vascular changes in the homocystinemic nonhuman primate and, possibly, in homocystinemic man.

Sulfinpyrazone also produced a significant direct reduction in the amount of specific ⁵¹Cr release from cultured human endothelial cells produced by 10 mM homocysteine after 24 hours of co-incubation. However, the high background makes the interpretation of that effect problematic, particularly when compared with the assessment of in vitro cell detachment (Table 2). No inhibition of homocysteine-induced endothelial detachment by sulfinpyrazone or one of its metabolites in vitro was observed. Muller (1980) and Weimann et al. (1980), in vitro, using two other assays, have also reported that sulfinpyrazone fails to protect cultured porcine or human endothelial cells from injury.

There are several possible explanations for the failure of sulfinpyrazone to protect the endothelium from homocysteine-induced detachment in vitro. The homocysteine concentration required to induce injury in vitro over 24 hours exceeds, by 10- to 100-fold, that required to produce endothelial injury in vivo, and any protective effect of sulfinpyrazone may be overwhelmed at this dosage. Alternatively, it is also possible that a metabolite of sulfinpyrazone other than the thioether might be responsible for the protective effect and, thus, would not be detected in our studies of cultured cells. In view of the limitations of in vitro studies, the failure to demonstrate a protective effect of sulfinpyrazone on endothelial cell detachment in vitro does not exclude efficacy in vivo.

Consideration must also be given to the possibility that sulfinpyrazone may produce its protective in vivo effect on endothelium by directly modifying homocysteine or its metabolism. The in vitro observation that sulfinpyrazone-mediated reduction in specific ⁵¹Cr release from cultured human endothelial cells induced by 10 mM homocysteine occurred only in the presence of sulfinpyrazone is consistent with the possibility that sulfinpyrazone may oxidize homocysteine directly. Alternatively, sulfinpyrazone could have altered other chemical pathways involv-
ing infused homocysteine, such as methylation to methionine, conversion to \( \alpha \)-ketobutyric acid, or \( \alpha \)-amino-\( \beta \)-butyric acid (Reddy and Wilcken, 1982).

On the basis of the present in vivo data and the reported protection of rat coronary arteriolar endothelial cells from stress-induced changes (Cairncross et al., 1979), we propose that the apparent in vivo efficacy of sulfinpyrazone in patients with vascular disease may be mediated, at least in part, through some protective effect on endothelial integrity.

We are indebted to Katie Stanness, Norma Jay, Tom Kirkman, Beverly Kariya-Jones, and Cellini Chen for technical assistance. We also wish to thank Drs. William Hazzard and Ramtrap Kushwaha of the Northwest Lipid Research Clinic for performing the lipid determinations.

This work was performed during the tenure of a Clinician Scientist Award of the American Heart Association, with funds contributed in part by American Heart Association of Washington.

Publication No. 2974-BCR from the Research Institute of Scripps Clinic, La Jolla, California.

Supported by Research Grants HL-29036, HL-18645, HL-21950, and RR-00166 from the U.S. Public Health Service.

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Received March 12, 1981; revised manuscript received August 5, 1983; accepted for publication September 14, 1983.

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INDEX TERMS: Arteriosclerosis • Endothelial injury • Homocystinemia • Sulfinpyrazone
Effect of sulfinpyrazone on homocysteine-induced endothelial injury and arteriosclerosis in baboons.

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Circ Res. 1983;53:731-739
doi: 10.1161/01.RES.53.6.731

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