

## The Thromboxane Receptor Antagonist S18886 but Not Aspirin Inhibits Atherogenesis in Apo E-Deficient Mice Evidence That Eicosanoids Other Than Thromboxane Contribute to Atherosclerosis

Antonio J. Cayatte, Yue Du, Jennifer Oliver-Krasinski, Gilbert Lavielle,  
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**Abstract**—Atherosclerosis involves a complex array of factors, including leukocyte adhesion and platelet vasoactive factors. Aspirin, which is used to prevent secondary complications of atherosclerosis, inhibits platelet production of thromboxane (Tx) A<sub>2</sub>. The actions of TxA<sub>2</sub> as well as of other arachidonic acid products, such as prostaglandin (PG) H<sub>2</sub>, PGF<sub>2α</sub>, hydroxyeicosatetraenoic acids, and isoprostanes, can be effectively antagonized by blocking thromboxane (TP) receptors. The purpose of this study was to determine the role of platelet-derived TxA<sub>2</sub> in atherosclerotic lesion development by comparing the effects of aspirin and the TP receptor antagonist S18886. The effect of 11 weeks of treatment with aspirin (30 mg · kg<sup>-1</sup> · d<sup>-1</sup>) or S18886 (5 mg · kg<sup>-1</sup> · d<sup>-1</sup>) on aortic root atherosclerotic lesions, serum levels of intercellular adhesion molecule-1 (ICAM-1), and the TxA<sub>2</sub> metabolite TxB<sub>2</sub> was determined in apolipoprotein E-deficient mice at 21 weeks of age. Both treatments did not affect body or heart weight or serum cholesterol levels. Aspirin, to a greater extent than S18886, significantly decreased serum TxB<sub>2</sub> levels, indicating the greater efficacy of aspirin in preventing platelet synthesis of TxA<sub>2</sub>. S18886, but not aspirin, significantly decreased aortic root lesions as well as serum ICAM-1 levels. S18886 also prevented the increased expression of ICAM-1 in cultured human endothelial cells stimulated by the TP receptor agonist U46619. These results indicate that inhibition of platelet TxA<sub>2</sub> synthesis with aspirin has no significant effect on atherogenesis or adhesion molecule levels. The effects of S18886 suggest that blockade of TP receptors inhibits atherosclerosis by a mechanism independent of platelet-derived TxA<sub>2</sub>, perhaps by preventing the expression of adhesion molecules whose expression is stimulated by eicosanoids other than TxA<sub>2</sub>. (*Arterioscler Thromb Vasc Biol.* 2000;20:1724-1728.)

**Key Words:** thromboxane receptor ■ atherosclerosis ■ aspirin ■ adhesion ■ thromboxane

Aspirin is the most widely used therapeutic agent for the secondary prevention of acute clinical complications of atherosclerotic cardiovascular disease.<sup>1</sup> Its therapeutic effect is widely attributed to its antiplatelet actions. However, there is little evidence that aspirin has any effects on the primary prevention or regression of atherosclerotic lesions that underlie acute cardiovascular events. Although newer antiplatelet agents have proven better in some respects than aspirin in the secondary prevention of cardiovascular events,<sup>2,3</sup> there is little clinical or experimental evidence that antiplatelet agents decrease atherosclerosis.<sup>4,5</sup> This is somewhat surprising, because in comparative studies with aspirin, other anti-inflammatory agents, including indomethacin,<sup>6</sup> cortisone,<sup>7</sup> colchicine,<sup>7</sup> and phenylbutazone,<sup>8</sup> reduce experimental atherosclerosis. Partly for these reasons, the role of platelets in atherogenesis is now questioned, whereas there is an accepted role for infiltration of inflammatory leukocytes in atherosclerotic lesion formation.<sup>9</sup>

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Although aspirin inhibits cyclooxygenase and therefore, the production of thromboxane (Tx) A<sub>2</sub>, it does not block the actions of other eicosanoids such as hydroxyeicosatetraenoic acids (HETEs)<sup>10,11</sup> and F<sub>2</sub>-isoprostanes,<sup>12</sup> whose production is increased in atherosclerosis. Because neither the production of these eicosanoids by inflammatory leukocytes or blood vessels nor their effects on the vasculature are prevented by aspirin, it is possible that they play a role in accelerating plaque growth that is not addressed by aspirin treatment. One possibility is suggested by the fact that thromboxane (TP) receptors are stimulated not only by TxA<sub>2</sub> but also by virtually all eicosanoids. Thus eicosanoids, including TxA<sub>2</sub>, may stimulate the expression of adhesion molecules<sup>13</sup> and as a result, increase monocyte adherence<sup>14</sup> and might accelerate plaque growth by that mechanism.<sup>15</sup> For these reasons, we

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compared the effect of a new TP receptor antagonist, S18886, with those of aspirin on lesion formation in apo E-deficient mice. S18886 is a potent, selective, TP receptor antagonist recently advanced into clinical development. This compound inhibits TP receptor-mediated vascular contractions with affinity constant values of  $\approx 9$  and TP receptor-mediated platelet aggregation with  $IC_{50}$  values of  $\approx 0.2 \mu\text{mol/L}$ .<sup>16-19</sup> We also evaluated the effect of treatment on circulating levels of intracellular adhesion molecule-1 (ICAM-1). The results indicate that although aspirin inhibits platelet-derived  $TxA_2$  production, it has no significant effect on atherosclerotic lesion formation or ICAM-1 levels. Both parameters were significantly decreased by S18886, suggesting an important role of eicosanoids other than  $TxA_2$  in promoting atherogenesis by their action at TP receptors.

## Methods

### Materials

The TP receptor antagonist S18886, which selectively blocks TP receptors, was obtained from the Institut de Recherches Internationales Servier, Suresnes, France. Aspegic, a water-soluble mixture of acetylsalicylic acid (36%) and the lysine salt of acetylsalicylate (64%) in powder form, was obtained from Laboratoires Synthelabo. The  $TxA_2$  mimetic and TP receptor agonist, U46619, was purchased from Cayman Chemical Co.

Anti-human ICAM-1 (IgG1), a mouse monoclonal antibody, was purchased from Endogen, and the alkaline phosphatase-conjugated goat anti-mouse IgG F(ab')<sub>2</sub> fragment and the nonimmune isotypic mouse purified IgG were obtained from Sigma Immunochemicals.

### Animal Protocol and Diet

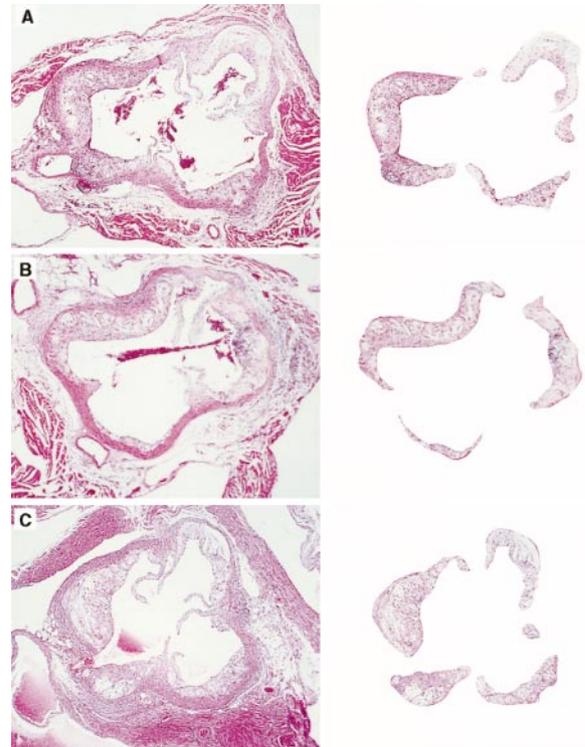
Female homozygous apo E-deficient mice (backcrossed for at least 10 generations to the C57BL/6J background) were obtained at 8 weeks of age from Jackson Laboratories (Bar Harbor, Me). The mice were fed normal mouse chow (Purina Certified Rodent Chow 5002) containing 4.5% fat and given free access to both food and water throughout the study. After 1 week of acclimatization, some mice were treated either with S18886 ( $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ) or with aspirin ( $30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ) added to the drinking water. The dose of S18886 was selected because studies in rats had illustrated that this dose completely prevented U46619-induced platelet aggregation (T. Verbeuren, personal communication, 1999). The dose of drug was calculated on the basis of the average consumption of water (5 mL/d) and the body weight, determined weekly. The mice were continued on treatment until 21 weeks of age, when they were killed by an overdose of sodium pentobarbital.

### Measurement of Serum Cholesterol, Soluble ICAM-1, and $TxB_2$

Blood samples were collected from within the thoracic cavity after cutting open the inferior vena cava before removal of the heart. After allowing the blood to clot and obtaining serum samples, cholesterol was measured enzymatically by using a kit from Sigma Diagnostics; soluble ICAM-1 was measured by using a kit from Endogen, Inc; and  $TxB_2$  levels were measured by using a kit from Cayman Chemical Co.

### Tissue Preparation and Quantification of Atherosclerotic Lesion Area

The hearts were removed immediately after the mice were killed, rinsed in cold PBS to remove traces of blood, and placed in formalin overnight. The hearts were sliced with a scalpel on a plane parallel to the tips of the atria at the base of the aortic root, according to a procedure described by Paigen et al.<sup>20</sup> The tissue was processed and embedded in paraffin for histological sectioning by conventional methods. Tissue cross sections,  $5 \mu\text{m}$  thick, were cut starting at the level of the aortic valve leaflets and continuing on until the valve cusps disappeared. For morphometric analysis of aortic root lesion



**Figure 1.** Representative photomicrographs of aortic root from control (A), S18886-treated (B), and aspirin-treated (C) apo E-deficient mice. Representative sections were chosen from mice whose average lesion area approximated the mean value for that group, and the sections shown were obtained at approximately the same level of the aortic root. Shown at the right of each cross section is the lesion area that was analyzed for that cross section. Original magnification was  $\times 40$ .

area, cross sections spaced  $50 \mu\text{m}$  apart were stained with hematoxylin-eosin and photographed at a magnification of  $\times 40$ . The images were scanned into a computer by using a Polaroid Sprint 35 scanner, and lesion area was determined on the computer-digitized images with NIH Image 3.0 software. For Figure 2A, the lesion area measured at each level of the tricuspid valve was analyzed and plotted with respect to distance from the initial cut through the valve. For Figure 1B, data collected from each of 5 sections taken from the entire length of the aortic valve was averaged and expressed as square millimeters per section as described by Paigen et al.<sup>20</sup> The analysis of lesions was done by an observer who was blinded to the treatment group.

### Human Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics Corp, San Diego, Calif, as cryopreserved cell suspensions and grown according to the manufacturer's seeding and culture protocol. Endothelial cells had a typical cobblestone morphology as assessed by phase-contrast microscopy and expressed von Willebrand factor antigen. Routine cell viability ( $>90\%$ ) was determined by trypan blue exclusion. Between the third and fifth passages, endothelial cells were used to seed 96-well Costar plates at a density of  $\approx 5000$  cells/well. Monolayers were used when the cells reached confluence, which required  $\approx 48$  hours.

After reaching confluence, HUVECs were cultured for an additional 6 hours with or without the  $TxA_2$  agonist U46619 and in some cases, in the presence of S18886 ( $1 \mu\text{mol/L}$ ) added 1 hour before the addition of U46619. This concentration of S18886 totally prevents U46619-induced arterial contractions and platelet aggregation.<sup>18</sup> These agents did not affect the physical appearance of the endothelial cell monolayer. After treatment, endothelial cell monolayers were placed on ice, washed, and incubated overnight in 2% paraformaldehyde with 0.05% Tween-20. Each experiment assessing ICAM-1

expression was repeated on at least 3 occasions, each time in triplicate.

### Endothelial Cell ICAM-1 Surface Expression

The expression of ICAM-1 on the surface of HUVECs was analyzed with an Ascent fluorometric plate reader (Laboratory Systems Corp) with a fluorescent ELISA to allow for the detection of ICAM-1 expressed on the cell surface. At confluence, monolayers were incubated for 90 minutes at room temperature with a saturating concentration (1  $\mu\text{g}/\text{mL}$ ) of either anti-human mouse monoclonal antibody to ICAM-1 or a nonimmune isotypic purified mouse IgG (Sigma Immunochemicals) used as a negative control. Unbound anti-ICAM-1 antibody and nonimmune IgG were removed by aspiration, and cells were incubated at room temperature for 30 minutes with a secondary alkaline phosphatase-tagged goat-anti mouse IgG antibody (Fab''<sub>2</sub> fragment) at a dilution of 1:1000 to minimize nonspecific binding. Nonadherent conjugated IgG was removed by washing, and bound ICAM-1 antibody was detected by addition of the fluorescent alkaline phosphatase substrate Attophos (JBL Scientific) at a concentration of 1  $\mu\text{mol}/\text{mL}$ . The reaction was stopped after a 30-minute incubation at room temperature by adding 33  $\mu\text{L}$  of 100 mmol/L EDTA to each well. Immunofluorescent intensity was detected by using sharp cutoff filters at an excitation wavelength of 444 nm and an emission wavelength of 555 nm, and ICAM-1 expression was quantified in fluorescent units after subtracting background values from blank wells with nonimmune IgG. ICAM-1 expression was calculated in arbitrary units of fluorescence and expressed as a percentage of expression relative to control groups.

### Monocyte Adherence to Endothelial Cells

Monocytic cells of the U937 cell line were labeled by incubation with calcein (5  $\mu\text{mol}/\text{L}$ , Molecular Probes) for 30 minutes at 37°C. Before the adhesion assay was performed, HUVECs were rinsed 3 times with cold PBS, and fluorescent labeled U937 cells were added to individual wells at a predetermined optimal concentration of  $2 \times 10^5$  cells/mL in RPMI 1640 medium (Sigma) containing 0.2% human serum albumin in a final volume of 100  $\mu\text{L}/\text{well}$ . Labeled U937 cells were allowed to adhere under static conditions to the monolayers for 30 minutes at 37°C in a humidified 95% O<sub>2</sub>/5% CO<sub>2</sub> atmosphere before removal of nonadherent cells by aspiration. The extent of adhesion was determined by directly measuring bound, fluorescent U937 cells to endothelial monolayers with the Ascent fluorometric plate reader with excitation and emission wavelengths of 485 and 530 nm, respectively. Adherence was quantified as the number of labeled monocytes adherent to endothelial cell monolayers.

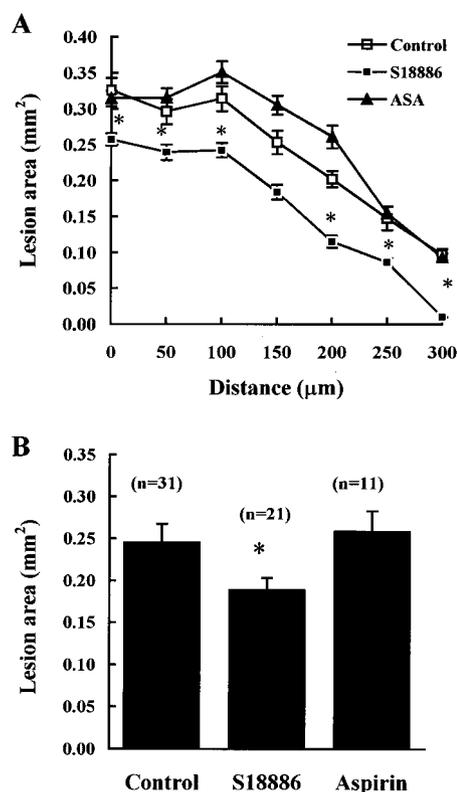
### Statistical Analysis

All data are presented as mean  $\pm$  SEM. ANOVA was used to analyze differences between the 3 groups; in addition, an unpaired 2-tailed Student's *t* test was performed to compare results between treatment groups, and statistical significance was assumed for probability values  $<0.05$ .

## Results

### S18886 Inhibits Aortic Atherosclerotic Lesion Area

Morphometric quantification of atherosclerotic lesion area on digitized cross sections of the aortic root, such as the representative image shown in Figure 1, was done in 21 apo E-deficient mice treated with S18886 (5  $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ) and 11 mice treated with aspirin (30  $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ) and compared with measurements in 31 untreated mice. For each of the sections analyzed every 50  $\mu\text{m}$  along the tricuspid aortic valve, the atherosclerotic lesion area was consistently and significantly less in the mice treated with S18886 than in control or aspirin-treated mice (Figure 2A). No significant difference was observed between control mice and those treated with aspirin. The average lesion area per cross section



**Figure 2.** Aortic root lesion area in control (n=31), S18886-treated (n=21), and aspirin-treated (n=11) apo E-deficient mice. A, Data for each individual cross section taken at 50- $\mu\text{m}$  intervals from the initial cut at the bottom to the top of the tricuspid valve. Data in B are mean  $\pm$  SEM of the average digitized lesion area in  $\text{mm}^2$  per cross section. S18886 ( $P < 0.04$ ), but not aspirin treatment, significantly reduced aortic lesion area (ANOVA). Asterisks indicate significant differences between individual points in the S18886-treated group from the control and aspirin-treated groups by Student's *t* test.

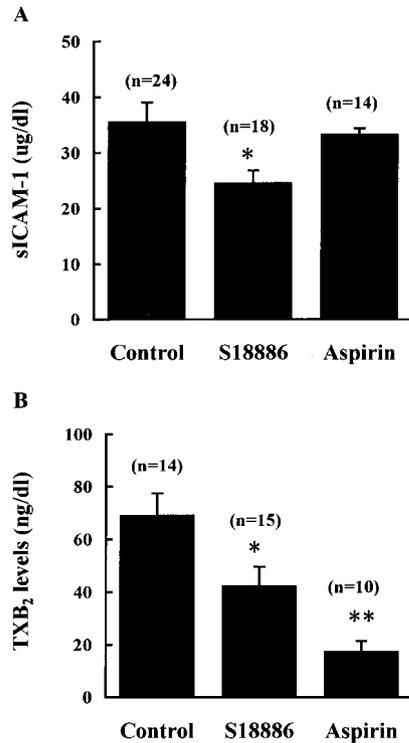
in control animals was  $0.24 \pm 0.02 \text{ mm}^2$  (Figure 2B). Treatment for 11 weeks with S18886 significantly reduced average lesion size to  $0.19 \pm 0.01 \text{ mm}^2$ , but treatment with aspirin for the same period had no significant effect on average lesion area ( $0.26 \pm 0.03 \text{ mm}^2$ ).

### Serum Soluble ICAM-1 and TxB<sub>2</sub> Levels

To investigate whether alterations in the adherence of blood monocytes to ICAM-1 on vascular endothelium could be one of the possible mechanisms by which treatment with S18886 reduced lesion size, the serum levels of soluble ICAM-1 were determined. As shown in Figure 3A, S18886-treated apo E-deficient mice had significantly lower levels of serum soluble ICAM-1 ( $24 \pm 2 \mu\text{g}/\text{mL}$ ) relative to control mice ( $35 \pm 3 \mu\text{g}/\text{mL}$ ). There was no significant effect of aspirin on ICAM-1 levels ( $33 \pm 1 \mu\text{g}/\text{mL}$ ). Treatment with S18886 and, to a significantly greater extent, aspirin, was associated with a significant reduction in serum TxB<sub>2</sub> levels (Figure 3B: control,  $69 \pm 8 \text{ ng}/\text{mL}$ ; S18886,  $42 \pm 8 \text{ ng}/\text{mL}$ ; and aspirin,  $17 \pm 4 \text{ ng}/\text{mL}$ ).

### S18886 Inhibits Increased ICAM-1 Expression and Adherence of U937 Cells Stimulated by U46619 in HUVECs

To determine whether S18886 could prevent the increased expression of ICAM-1 on endothelial cells that has been



**Figure 3.** Serum ICAM-1 (A) and TxB<sub>2</sub> (B) levels. Data are mean±SEM. Serum ICAM-1 level in S18886-treated apo E-deficient mice (n=18) was significantly less than in control ( $P<0.02$ , n=24) as well as aspirin-treated ( $P<0.01$ , n=14) mice. Serum TxB<sub>2</sub> in S18886-treated ( $P<0.02$ , n=15) and aspirin-treated ( $P<0.01$ , n=10) mice was significantly different from that in controls (n=14). The TxB<sub>2</sub> level in aspirin-treated mice was also significantly less than that in S18886-treated mice ( $P<0.02$ ).

reported to occur in response to TxA<sub>2</sub> receptor stimulation, the ability of the TP receptor antagonist was tested against the TxA<sub>2</sub> agonist U46619 in HUVECs. TP receptor stimulation increased endothelial cell ICAM-1 expression in a concentration-dependent fashion, with a significant increase in expression of 150±17% with 0.1 μmol/L and of 169±34% with 1 μmol/L U46619 (Figure 4A). The effect of U46619 (1 μmol/L) was completely prevented by S18886 (1 μmol/L).

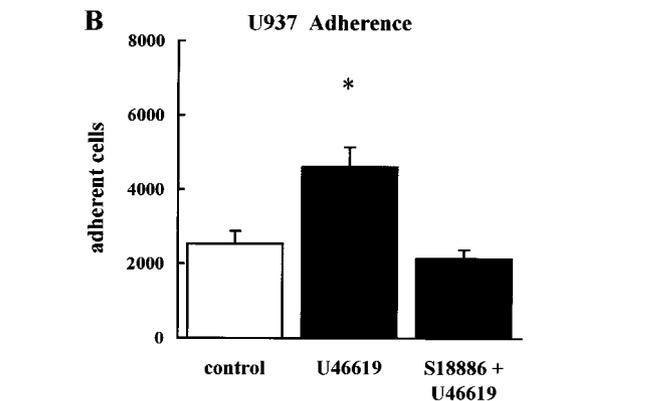
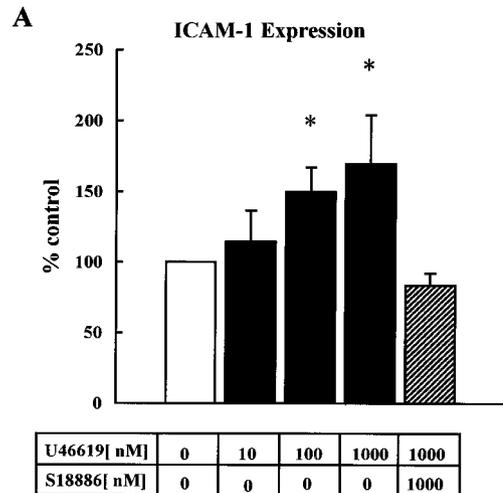
To determine whether the inhibition of ICAM-1 expression by S18886 affected monocyte adherence to endothelial cells, we examine its effect on the adherence of calcein-labeled U937 monocytic cells to HUVEC monolayers stimulated with 1 μmol/L U46619. TP receptor stimulation with U46619 significantly increased basal adherence from 2533±333 to 4600±529 adherent cells (Figure 4B), which was completely prevented by S18886 (2133±240).

### Serum Cholesterol Measurements and Body Weight

S18886- or aspirin-treated apo E-deficient mice showed no significant changes in serum cholesterol levels or body weight relative to control mice (the Table), suggesting that S18886 had no metabolic influence to explain its effect on atherosclerotic lesion development.

### Discussion

The important findings in this study are that (1) a TP receptor antagonist, S18886, decreased the development of atherosclerosis and serum levels of ICAM-1 in the apo E-deficient



**Figure 4.** Effect of U46619 and S18886 on ICAM-1 expression (A) and U937 adherence to cultured HUVECs (B). Expression of ICAM-1 is denoted as a percentage of control and U937 adherence as the number of calcein-labeled adherent cells. ICAM-1 expression ( $P<0.05$ ) and U937 adherence ( $P<0.01$ ) were significantly stimulated by 1 μmol/L U46619, an effect that was prevented by S18886 (1 μmol/L for ICAM-1 expression and 100 μmol/L for U937 adherence). Data are mean±SEM of the values from 3 experiments, each performed in triplicate, and between-group analysis was performed by using single-factor ANOVA.

mouse; (2) despite inhibiting serum TxA<sub>2</sub> production, which is derived primarily from platelets, aspirin decreased neither ICAM-1 levels nor atherosclerosis; and (3) S18886 blocked the increased expression of ICAM-1 in HUVECs stimulated by a TP receptor agonist, U46619. Although our findings were made at only one time point and dose of treatment, they suggest that TP receptors play an important role in the development of atherosclerosis in the apo E-deficient mouse and, together with the lack of effect of aspirin, that eicosanoids other than platelet-derived TxA<sub>2</sub> are responsible.

### Effect of S18886 and Aspirin on Metabolic Parameters

Parameter	Control	S18886	Aspirin
Body weight, g	22±1	22±1	21±1
Heart weight, mg	114±3	117±4	114±3
Serum cholesterol, mmol/L	11±0.4	12±0.9	11±0.8

Data are mean±SEM for the 3 groups of control, S18886-, and aspirin-treated apo E-deficient mice. The numbers of samples for each group of mice are given in Figure 2. No significant differences occurred as a result of treatment.

The infiltration of monocytes is a key “inflammatory” event in early atherogenesis.<sup>9</sup> The increased expression of endothelial cell adhesion molecules such as ICAM-1, which can be stimulated by TP receptor agonists, is one potential mechanism by which TP receptors could be involved in promoting lesion formation. Indeed, in cultured HUVECs, S18886 prevented the increased expression of ICAM-1 stimulated by U46619 as well as the adherence of mononuclear cells. In addition, the decrease in serum ICAM-1 levels in apo E-deficient mice treated with S18886 is consistent with the regulation of adhesion molecules as a mechanism by which the drug could influence atherosclerosis. The fact that aspirin decreased neither ICAM-1 levels nor lesion formation further supports this suggestion.

Although our study does not identify the potential eicosanoids involved, there are several candidates, all of which are capable of stimulating TP receptors and whose production is known not to be inhibited by aspirin. One possibility is that F<sub>2</sub>-isoprostanes, which are nonenzymatic oxidation products of arachidonic acid that are capable of stimulating TP receptors, are involved in promoting atherosclerosis. Consistent with this suggestion is that F<sub>2</sub>-isoprostane serum levels are elevated in apo E-deficient mice and that treatment with vitamin E reduced both lesion size and levels of F<sub>2</sub>-isoprostanes.<sup>12</sup> Another possibility is HETEs, which either are products of lipoxygenase or can be formed by nonenzymatic lipid peroxidation in endothelial cells and leukocytes. HETEs are recognized to be increased in atherosclerosis,<sup>10,11</sup> to participate in inflammation,<sup>21</sup> and together with isoprostanes have been localized in atherosclerotic plaques.<sup>22</sup> HETEs,<sup>10,23</sup> like isoprostanes, are known to activate vascular TP receptors and thus could explain the effect of S18886.

By inhibiting the formation of prostaglandins and increasing the availability of more arachidonic acid, it is possible that aspirin could actually increase the formation of the above-mentioned eicosanoids and their stimulation of TP receptors. Interestingly, 15-HETE may be formed by the inducible isoform of cyclooxygenase, which is expressed in atherosclerotic plaques, even after aspirin treatment.<sup>24</sup> To some extent, S18886, like TP receptor antagonists in general, also reduced TxB<sub>2</sub> levels. However, unlike with aspirin, this effect of S18886 was not caused by inhibiting cyclooxygenase but presumably by interrupting the positive feedback exerted on platelet production of TxA<sub>2</sub>, which is mediated by TP receptors stimulated by TxA<sub>2</sub> released during platelet aggregation.<sup>19</sup> If, as the data presented here suggest, eicosanoids whose effects are not blocked by aspirin promote atherosclerosis, then there would be increased rationale for the clinical use of TP receptor antagonists like S18886. Of course, TP receptor antagonists may also provide the additional therapeutic benefit of blocking the actions of platelet-derived TxA<sub>2</sub>, thus allowing the normal production of prostacyclin, inhibiting platelet aggregation, and also favoring the secondary prevention of acute thrombotic complications of atherosclerotic cardiovascular disease.<sup>3,5</sup>

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