The Thromboxane A2 Receptor Antagonist S18886 Prevents Enhanced Atherogenesis Caused by Diabetes Mellitus

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**Background**—S18886 is an orally active thromboxane A2 (TXA2) receptor (TP) antagonist in clinical development for use in secondary prevention of thrombotic events in cardiovascular disease. We previously showed that S18886 inhibits atherosclerosis in apolipoprotein E–deficient (apoE−/−) mice by a mechanism independent of platelet-derived TXA2. Atherosclerosis is accelerated by diabetes and is associated with increased TXA2 and other eicosanoids that stimulate TP. The purpose of this study was to determine whether S18886 lessens the enhanced atherogenesis in diabetic apoE−/− mice.

**Methods and Results**—Diabetes mellitus was induced in apoE−/− mice with streptozotocin and was treated or not with S18886 (5 mg · kg−1 · d−1). After 6 weeks, aortic lesion area was increased >4-fold by diabetes in apoE−/− mice, associated with similar increases in serum glucose and cholesterol. S18886 largely prevented the diabetes-related increase in lesion area without affecting the hyperglycemia or hypercholesterolemia. S18886 prevented deterioration of endothelial function and endothelial nitric oxide synthase expression, as well as increases in intimal markers of inflammation associated with diabetes. In human aortic endothelial cells in culture, S18886 also prevented the induction of vascular cell adhesion molecule-1 and prevented the decrease in endothelial nitric oxide synthase expression caused by high glucose.

**Conclusions**—The TP antagonist inhibits inflammation and accelerated atherogenesis caused by diabetes, most likely by counteracting effects on endothelial function and adhesion molecule expression of eicosanoids stimulated by the diabetic milieu. (Circulation. 2005;112:3001-3008.)

Key Words: atherosclerosis ■ diabetes mellitus ■ inflammation ■ nitric oxide synthase ■ thromboxane

Atherosclerosis is an inflammatory disease of the intima of large arteries that is promoted by high serum cholesterol and in which various types of cells, including monocytes/macrophages, endothelial cells, smooth muscle cells, and platelets, exert a complex array of interactions. Nowhere is atherosclerosis accelerated to a greater degree than by diabetes mellitus, in which hyperglycemia and hyperlipidemia promote the inflammatory process. In apolipoprotein E–deficient (apoE−/−) mice, streptozotocin-induced diabetes causes a 3- to 5-fold increase in atherogenesis.1–3 In this mouse model, the mechanism of the accelerated atherosclerosis has been attributed to advanced glycation end products (AGEs)1 and angiotensin II2–3 in addition to the increased hyperglycemia and hyperlipidemia observed.

In previous work from this laboratory, it was shown that a thromboxane A2 (TXA2) receptor (TP) antagonist, S18886, significantly inhibits atherosclerosis in apoE−/− mice.4 Because aspirin had no effect, the mechanism was shown to be independent of platelet-derived TXA2 production. A similar effect of S18886 was observed in apobec-1/LDL receptor double-knockout mice.5 Apo E−/− mice genetically deficient in TP receptors also demonstrated attenuated atherosclerosis,6 confirming the important role that TP receptors play in atherogenesis. In diabetes mellitus, high glucose promotes the production of eicosanoids that are believed to contribute to the disease process.7 For instance, eicosanoids produced in endothelial cells exposed to high glucose levels contribute to adhesion molecule expression, and TP receptors mediate the effect.8 In addition, TP receptors mediate to a striking degree the inflammatory response to cytokines, as demonstrated by the effect of TP antagonists.9,10 Therefore, the present studies were undertaken to determine whether S18886 prevents the accelerated atherogenesis observed in diabetic apoE−/− mice and to investigate the mechanisms by which it does so in
studies of human aortic endothelial cells (HAECs). We found that the TP antagonist abrogated the 4-fold increase in atherosclerotic lesions as well as the intimal inflammation caused by diabetes despite having no effect on hyperglycemia and hypercholesterolemia. In HAECs, S18886 prevented the enhanced vascular cell adhesion molecule-1 (VCAM-1) expression and the decrease in endothelial nitric oxide synthase (eNOS) expression observed in cells exposed to high glucose, indicating that eicosanoids generated by the endothelium exposed to hyperglycemia stimulate TP receptor-mediated endothelial dysfunction, increases in adhesion molecule expression, and acceleration of atherogenesis in diabetes.

Methods

Materials
The TP antagonist S18886 was obtained from the Institut de Recherches Servier, Suresnes, France. Streptozotocin and other reagents were obtained from Sigma Chemical Co.

Animal Protocol and Diet
Female homozygous apoE−/− mice (backcrossed for at least 10 generations to the C57BL/6 background) were obtained at 7 weeks of age from Jackson Laboratories (Bar Harbor, Maine). The mice were fed normal mouse chow (Purina Certified Rodent Chow 5002) continuously with 95% oxygen/5% carbon dioxide in a physiological stirrups in organ chambers and were maintained at 37°C and bubbled with intact endothelium were mounted on 0.005-inch-diameter metal stirrups in organ chambers and were maintained at 37°C and bubbled with 5% carbon dioxide in a physiological salt solution of the following composition (mmol/L): KCl 4.7, CaCl2 2.5, NaCl 118.3, KH2PO4 1.2, MgSO4 0.6, NaHCO3 25, and dextrose 5.5. Rings were stretched incrementally to an optimal tension of 2 g during 1 hour with repeated washing. After a 30-minute equilibration, the rings were contracted to physiological salt solution containing 50 mmol/L KCl and rinsed. After another 30-minute equilibration, rings were contracted with phenylephrine to ~1 g. Relaxation to cumulative half-logarithmic concentrations of acetylcholine (10−5 to 10−3 mol/L) or sodium nitroprusside (10−10 to 10−6 mol/L) was determined. Relaxation is expressed as percent decrease in the phenylephrine-induced force. To study the acute effect of S18886, some rings from untreated diabetic animals were exposed for 30 minutes to S18886 (1 μmol/L). Some rings were then rinsed and contracted again with phenylephrine, and relaxation was elicited to sodium nitroprusside.

HAEC Culture
HAECs were obtained from Cambrex, Walkersville, Md, as cryopreserved cell suspensions and were cultured in endothelial cell growth medium-2 containing 2% fetal bovine serum and Single-Quots. Cells between the fifth and eighth passages were used for experiments. After they reached confluence, the cells were cultured with 0.5% fetal bovine serum endothelial cell basal medium-2 and pretreated with S18886 (1 or 10 μmol/L) for 1 hour, then stimulated in the continued presence of S18886 with U46619 (0.4 ng/mL) for 18 hours or exposed to d-glucose (30 mmol/L) or d-mannitol (25 mmol/L) plus d-glucose (5 mmol/L) for 3 days. In some cultures, N′-nitro-L-arginine methyl ester (L-NAME) (1 μmol/L) was added to the medium for the 3-day period. To study the effect of S18886 (1 μmol/L, 3 days) on another chronic model of endothelial inflammation, HAECs were stimulated for 3 days with tumor necrosis factor-α (TNF-α) (0.4 ng/mL). VCAM-1 and eNOS expression were determined by immunoblotting by standard methods. Anti-human VCAM-1 and eNOS antibodies were purchased from Santa Cruz Biotecnology (Santa Cruz, Calif) and Cell Signaling Technology (Beverly, Mass), respectively.

Superoxide Anion Measurement
To determine whether S18886 scavenges free radicals, the effects of the compound on reduction of acetylated cytchrome c (80 μmol/L) caused by hypoxanthine (100 μmol/L) and xanthine oxidase (0.5 μmol/mL) were assessed spectrophotometrically as previously described.

Statistical Analysis
All data are presented as mean±SEM. ANOVA was used to compare data from the 3 groups of mice with Dunn or Tukey-Kramer post hoc tests. Logarithmic transformation of atherosclerotic lesion area was performed. Statistical evaluation of the concentration-dependent inhibition of acetylcholine-induced relaxation in cultured endothelial cells and of the in vivo rat aortic blood flow response was performed. The results of 3 experiments were presented as mean±SEM. The α level for significance was set at 0.05.
response curve to acetylcholine (10⁻⁴ to 10⁻⁵ mol/L) in the 3 animal groups was performed with a 1-way ANOVA with repeated measures and post hoc analysis with Bonferroni multiple comparison test. A probability value of <0.05 was considered statistically significant.

Results

Effect of Diabetes and S18886 on Metabolic Parameters in ApoE⁻/⁻ Mice

Untreated diabetic apoE⁻/⁻ mice showed significant decreases in body and heart weight as well as a significant 3-fold increase in blood glucose and a 2.5-fold increase in serum cholesterol compared with nondiabetic apoE⁻/⁻ mice (Table). Treatment with S18886 did not cause significant changes in body and heart weight or in blood glucose or serum cholesterol relative to untreated diabetic mice, suggesting that the effects of S18886 on atherosclerotic lesion development are not related to any metabolic influence or alteration in severity of diabetes.

S18886 Decreases Aortic Atherosclerotic Lesion Area in Diabetic ApoE⁻/⁻ Mice

Total atherosclerotic lesion area was quantified in 21 nondiabetic apoE⁻/⁻ mice, 14 untreated diabetic mice, and 20 diabetic apoE⁻/⁻ mice treated with S18886. Diabetes produced a 5-fold increase in aortic atherosclerotic lesion area compared with nondiabetic animals (568±103 versus 132±17×10⁻² μm²; *P<0.0001). Treatment with S18886 significantly reduced the average lesion area to 284±38 (P<0.05 versus nondiabetic or versus untreated diabetic animals; Figure 1).

Effect of S18886 on Endothelium-Dependent Relaxation

Endothelium-dependent relaxations to acetylcholine in aorta of diabetic apoE⁻/⁻ mice were significantly decreased compared with nondiabetic apoE⁻/⁻ mice (Figure 2A). In aortic rings from diabetic apoE⁻/⁻ mice treated with S18886, relaxations were not significantly different from those in nondiabetic apoE⁻/⁻ mice. In addition, when aortas from untreated diabetic animals were exposed to S18886 (1 μmol/L, 30 minutes), relaxations to acetylcholine were restored to those that were not significantly different from nondiabetic animals (Figure 2A). Relaxations of apoE⁻/⁻ mouse aortic smooth muscle to sodium nitroprusside were not affected by diabetes or either acute or chronic treatment with S18886 (Figure 2B).

To consider whether a potential direct antioxidant action of S18886 could mediate its effect on endothelial function, the ability of the compound to scavenge oxidants generated by xanthine oxidase was tested. In concentrations from 1 to 100 μmol/L, S18886 did not affect the reduction of cytochrome c by hypoxanthine and xanthine oxidase, indicating that even in high concentrations, there was no demonstrable radical scavenging activity (Figure 2C).
Effect of S18886 on Vascular eNOS, Inflammation, and Oxidant Stress

To determine whether changes in expression of eNOS may have contributed to the alteration of endothelial function observed in diabetic apoE\(^{-/-}\) mice, immunohistochemical staining was performed simultaneously on cross sections of the aorta from the 3 groups of mice and scored semiquantitatively. Compared with nondiabetic apoE\(^{-/-}\) mice, eNOS staining of areas of the intima that were not involved with atherosclerotic lesions was significantly decreased in diabetic apoE\(^{-/-}\) mice, and this decrease was prevented by treatment with S18886 (Figure 3).

To assess the effect of the TP antagonist on markers of vascular inflammation and oxidant stress (Figure 3), aortic cross sections were stained for VCAM-1, nitrotyrosine, and AGEs. In aortic intima not involved with atherosclerotic lesions, each of these parameters was significantly increased in diabetic apoE\(^{-/-}\) mice compared with their nondiabetic littermates, and treatment with S18886 prevented the increase (Figure 3).

Effect of S18886 on eNOS Expression in HAECs Exposed to U46619 or Elevated Glucose

To identify potential mechanisms by which S18886 might affect atherosclerotic lesion development in diabetic apoE\(^{-/-}\) mice, the effects of S18886 were examined in HAECs. HAECs exposed to the TP agonist U46619 (1 \(\mu\)mol/L, 18 hours) showed a significant decrease in eNOS protein expression that was prevented by S18886 (1 \(\mu\)mol/L), indicating that TP activation can directly decrease eNOS expression (Figure 4A). In HAECs activated with the proinflammatory cytokine TNF-\(\alpha\) (0.4 ng/mL) for 3 days, eNOS expression was also significantly decreased (Figure 4B). S18886 (1 \(\mu\)mol/L, 3 days) significantly increased eNOS expression and prevented the decrease caused by TNF-\(\alpha\) (Figure 4B).

The effect of S18886 on eNOS protein expression was also determined in HAECs cultured for 3 days in control (5 mmol/L) or elevated (30 mmol/L) glucose. Elevated glucose significantly decreased eNOS expression compared with HAECs exposed to control glucose (Figure 4C). S18886 (1 to 10 \(\mu\)mol/L) increased the expression of eNOS in HAECs exposed to either control or elevated glucose for 3 days and prevented the decrease in eNOS expression caused by elevated glucose (Figure 4B).

Effect of S18886 on VCAM-1 Expression in HAECs Exposed to U46619 or Elevated Glucose

As previously reported for human umbilical vein endothelial cells, U46619 (1 \(\mu\)mol/L, 18 hours) increased expression of VCAM-1 in HAECs, and the increase was completely prevented by treatment with S18886 (1 \(\mu\)mol/L; data not shown). Exposure to elevated glucose (30 mmol/L, 3 days) also significantly increased VCAM-1 expression (Figure 5A). S18886 (1 to 10 \(\mu\)mol/L) decreased VCAM-1 expression in HAECs exposed to normal glucose and prevented the increase caused by high glucose exposure (Figure 5A). Cells exposed to mannitol (25 mmol/L) did not demonstrate an increase in VCAM-1, indicating that elevated glucose increases VCAM-1 independently of hyperosmolarity, and S18886 decreased VCAM-1 expression as it did in cells exposed to normal glucose (Figure 5A).

Because of the effects of S18886 on both VCAM-1 and eNOS expression, we performed studies to determine whether...
S18886 might inhibit VCAM-1 expression by increasing eNOS expression and NO production, which is known to inhibit VCAM-1 expression. HAECs exposed to 5 or 30 mmol/L glucose for 3 days were treated with L-NAME, S18886, or both. L-NAME (1 mmol/L) increased VCAM-1 expression in control glucose (5 mmol/L) to approximately the same level as high glucose (Figure 5A). S18886 significantly inhibited VCAM-1 expression in either 5 or 30 mmol/L glucose, and it did so to a similar extent in the presence or absence of L-NAME (Figure 5B).

Discussion

It has been demonstrated previously that S18886 or genetic deletion of TP receptors attenuates the development of atherosclerosis in nondiabetic apoE−/− mice, indicating an important role for TP receptors in the progression of atherosclerosis. The new findings in the present study show that S18886 largely prevents the impressive acceleration of atherosclerosis caused by type 1 diabetes induced by streptozotocin and prevents the deterioration in endothelium-dependent relaxation associated with diabetes. In addition, the TP antagonist largely prevented the decrease in eNOS protein and increase in intimal inflammatory markers associated with diabetes. As in the aorta in vivo, in HAECs exposed to elevated glucose in vitro, S18886 ameliorated the decrease in eNOS and increase in VCAM-1 expression that occurred, demonstrating direct effects of the TP antagonist on endothelial factors that potentially exacerbate atherogenesis in diabetes.

S18886 was developed as a highly specific, high-affinity TP antagonist. Binding studies showed that the drug displaces the binding of [3H]SQ29548 on human platelet membranes with a Kᵢ value of 0.65 nmol/L, and the Kᵢ value for binding of [3H]S18886 to human platelet membranes averaged 0.96 nmol/L.13 Further binding studies showed that S18886 did not interfere with binding of appropriate ligands to a series of receptors, channels, and enzymes. Furthermore, in extensive pharmacological investigations the compound only blocked TP receptor-mediated reactivity, such as those mediating vascular contractions or platelet responses (T.J.V., personal communication, 1994). Of particular interest to studies of atherosclerosis was the fact that S18886 did not react with other P receptors, such as IP or DP receptors, and thus preserved or even potentiated the vascular relaxation to prostacyclin and prostaglandin D₃.14

The effect of S18886 on atherogenesis was independent of any significant change in the large increases in hyperglycemia and hypercholesterolemia that accompanies streptozotocin-induced diabetes in the apoE−/− mouse.1 This suggests a potential local effect of the TP antagonist on lesion formation within the diabetic vascular wall that attenuates the atherogenic effects of the marked hyperglycemia and hypercholesterolemia. These atherogenic factors may accelerate atherosclerosis in diabetes by multiple mechanisms that include increased production of cytokines and growth factors. Angiotensin II is recognized as one such factor that accelerates diabetic vascular disease, and indeed, angiotensin-converting enzyme inhibition2 and angiotensin II receptor blockade3 potently inhibit atherosclerotic lesion development in diabetic apoE−/− mice. Angiotensin II stimulates vascular inflammation and eicosanoid production15, and therefore TP may account for part of its actions. However, S18886 has no effect on responses mediated by a number of G protein–linked receptors other than TP, including the AT₁ receptor (T.J.V., personal communication, 1994). This suggests a link between the proatherogenic mechanisms stimulated by angiotensin II and eicosanoids that stimulate TP.

Evidence that S18886 prevented the actions of local atherogenic factors also was obtained from studies of VCAM-1 expression that was increased by diabetes and decreased by the TP antagonist in aortic intima not involved by lesions. AGEs form as the result of adduction reactions of glucose with proteins during diabetes, atherosclerosis, and aging and were previously
reported to be present in apoE−/− mouse aorta and to be upregulated by diabetes,1 as also shown in this study. The attenuation of AGEs in aortic intima of diabetic apoE−/− mice in the face of unchanged hyperglycemia most likely indicates a strong effect of TP receptor blockade on the inflammatory state that stimulates AGE formation in atherosclerosis and aging even in the absence of hyperglycemia.

In addition, we investigated oxidant effects of diabetes on the aortic intima by immunostaining for nitrotyrosine. This oxidant indicator was also increased by diabetes and attenuated by the TP antagonist. Because of the specificity of S18886 and the lack of any demonstrable antioxidant activity of the drug, our results showing attenuation of VCAM-1, AGEs, and nitrotyrosine are most compatible with an antiinflammatory effect of TP receptor blockade that counters the effect of the diabetic milieu.

Eicosanoid production is also well known to be increased in diabetes, likely as a result of the increased vascular inflamma-


The role of TP in regulating the endothelial cell response to diabetes is also indicated by the ability of S18886 to prevent changes in eNOS and VCAM-1 expression induced directly by elevated glucose in cultured HAECs. The effect of elevated glucose on eNOS in cultured endothelial cells is controversial, with evidence of both increases and decreases having been presented.23–26 In aortic intima not involved with atherosclerotic lesions and in HAECs exposed to elevated glucose, we observed a significant reduction in eNOS expression similar to that ascribed by others to the activation of an activator protein-1 (AP-1) site in the eNOS promoter in HAECs exposed to elevated glucose.26 The fact that S18886 prevented the decrease both in vivo and in vitro indicates the role of TP in mediating the effect of the diabetic milieu on eNOS protein expression. We also found that S18886 prevents the decrease in eNOS expression caused by TNF-α, which is also known to activate AP-1 in endothelial cells.27 A potential interaction of TP receptors and AP-1 occurs at the level of TP receptor expression where an AP-1 regulatory region of the TPβ gene, the isoform that is expressed in endothelium,28,29 AP-1 is also a key transcription factor involved in the expression of VCAM-1.30 This suggests that the effects of elevated glucose on both eNOS and VCAM-1 transcription may be via similar mechanisms. The new finding here is that TP receptors play an important role in regulating the expression of 2 proteins whose function is important in atherogenesis and is altered by high glucose. High glucose also is known to increase the expression of cyclooxygenase-2 and lipoxygenase in cultured HAECs,31 and our study suggests that these might contribute to the eicosanoids that affect adhesion molecule and eNOS expression by stimulating TP receptors.

Because NO is known to regulate atherogenesis26,32 and adhesion molecule expression, the possibility exists that the decrease in eNOS expression contributes to the increase in VCAM-1 expression caused by elevated glucose. Indeed, the NOS inhibitor L-NAME increased VCAM-1 expression in a manner similar to that of elevated glucose. However, at least in culture, the effect of S18886 on VCAM-1 expression appears to be independent of the enhanced eNOS expression or of the potential changes in NO bioactivity it may have caused because the effect of S18886 was similar in the HAECs treated or not with L-NAME. It is difficult to determine the functional effects of the decrease in eNOS expression that we observed in vivo. Because S18886 in vitro can acutely normalize endothelium-dependent relaxation of the aorta in response to acetylcholine, the effects of endogenous eicosanoids on TP receptors in the aortic endothelium are apparently functionally more important than changes in eNOS expression. Nevertheless, it is possible that the improved NO bioactivity observed in the isolated aorta of diabetic apoE mice treated with S18886 in vivo may contribute to the beneficial effect observed on atherogenesis. The studies in HAECs presented here demonstrate that the local activity of eicosanoids on TP in endothelial cells exposed to elevated glucose is sufficient to promote events similar to those in vivo that are thought to contribute to atherogenesis.

In summary, our study indicates a potential therapeutic role for TP receptor antagonism in the accelerated atherogenesis that is associated with diabetes. Although one cannot be certain that the factors that exacerbate atherosclerosis in diabetes do so by...
mechanisms specific only to that disease, one can conclude that the greater effect of the TP antagonist in the diabetic apoE<sup>−/−</sup> mice studied here compared with its effect in nondiabetic apoE<sup>−/−</sup> mice nearly completely countered those mechanisms that account for the greatly accelerated course of atherosclerosis. It is likely that this beneficial effect is related to preventing the TP-mediated deterioration in eNOS expression and endothelial function and the increased inflammatory gene expression and oxidant stress that occur in the response of endothelial cells to the diabetic milieu.

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References

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