Experimental Sympathetic Activation Causes Endothelial Injury in the Rabbit Thoracic Aorta via $\beta_1$-Adrenoceptor Activation

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Sympathetic activation appears to accelerate the development of atherosclerosis, an effect that may be inhibited by $\beta$-receptor blockade. It is unclear, however, which mechanisms mediate this effect. In view of the significance attached to endothelial injury in the initial phases of atherogenesis, we decided to test whether sympathetic activation might lead to an increase in endothelial injury. Chloralose anesthesia was used to induce sympathetic activation and the presence of intracellular IgG as a criterion of endothelial cell injury. The $\beta_1$-selective $\beta$-blocker metoprolol was used to evaluate if the effect(s) of sympathetic activation might be mediated by $\beta_1$-adrenoceptors. In normal rabbits, the frequency of injured endothelial cells in unbranched areas of the thoracic aorta was 0.23%, compared with 1.93% in circumstomal areas. Chloralose anesthesia caused significant increases in blood pressure, heart rate, and plasma norepinephrine, that is, caused sympathetic activation, and led to an approximately fivefold increase in the number of injured cells both in unbranched and in circumstomal areas. This increase was totally inhibited by metoprolol pretreatment, indicating that it was mediated by $\beta_1$-receptors. These observations suggest one possible mechanism that may connect sympathetic activation with atherogenesis and explain why $\beta$-blockade protects against atherosclerosis. (Circulation Research 1990;67:1027-1034)

The significance of behavioral and psychosocial factors as regards the development of clinical complications in atherosclerosis has been discussed for decades (for reviews, see References 1 and 2 and references therein). More recently, a special behavioral pattern, the type A behavior, has been discussed as a potential risk factor for coronary heart disease.4,5 The significance of these factors has gained support from recent studies in cynomolous monkeys, indicating that psychosocial stress accelerates the development of coronary atherosclerosis in individuals with a certain type of behavior.5 This effect appeared to involve an increase in sympathetic nervous activity, because it was inhibited by $\beta$-adrenoceptor blockade.6 Several other studies in various experimental animal systems have also shown an antiatherosclerotic effect of $\beta$-blockade, including the $\beta_1$-adrenoceptor–selective $\beta$-antagonist metoprolol.7 It is unclear, however, what mechanistic link connects sympathetic activation, $\beta$-receptors, and the development of atherosclerosis. In view of the central role attributed to endothelial injury or dysfunction in atherogenesis (see References 8 and 9 and references therein), we wanted to test the hypothesis that sympathetic activation leads to endothelial dysfunction and that this effect might be inhibited by $\beta_1$-adrenoceptor blockade.

It is still unclear, however, what form endothelial injury might take. The presence of outright endothelial denudation preceding atherogenesis has been difficult to establish. It has recently been proposed that functional changes in the endothelium are involved in atherogenesis.10-13 The presence of dying and regenerating endothelial cells may be a basis for endothelial dysfunction. It has been observed that dying cells, unable to maintain their intracellular composition, may reside in the arterial endothelium for days.14 Such cells are unable to exclude plasma proteins and can be visualized through their content of IgG bound to vimentin.14-16

We decided to use the above technique to study the effects of activation of the sympathetic nervous system on endothelial integrity even though it is difficult to obtain a stable, persistent, and reproducible sympathetic activation in conscious animals. However, we observed that chloralose anesthesia in a stable and reproducible way increased heart rate, blood pressure, and plasma levels of norepinephrine, thus indicating...
sympathetic activation. Using this model, we were able to compare the frequency of IgG-positive endothelial cells in a group of conscious undisturbed rabbits with that in rabbits (with or without β-blockade) with increased sympathetic activity.

Materials and Methods

The animals used were male New Zealand White rabbits, which were allowed to acclimatize in the animal department for at least 1 week before the experiments. They were fed standard rabbit chow (Ewos K1), and body weight ranged from 2.0–3.8 kg.

Experimental Procedure

The experimental procedure for the two chloralose-treated groups is outlined in Figure 1a. In the conscious rabbits, the central ear artery was cannulated for blood pressure and heart rate recordings, and a cannula was also inserted in the marginal ear vein. After 15 minutes of baseline recordings, the group treated with chloralose and metoprolol received a bolus injection of metoprolol (0.3 mg/kg i.v.), followed by infusion (0.15 mg/kg · hr) of the drug dissolved in Ringer’s glucose. The chloralose-treated animals were infused with the Ringer’s glucose. After 1 hour of blood pressure and heart rate recordings, anesthesia was induced with intravenous metohexitol (Brietal, Eli Lilly and Co., Indianapolis). A tracheotomy was performed, and a ventilator was connected to the tracheal cannula, after which a slow intravenous administration of chloralose (75 mg/kg, Aldrich-Chemie, Steinheim, FRG) was started. Pancuronium bromide (Pavulon, Organon Inc., Oss, The Netherlands) was added to the chloralose solution (2 mg) and to the Ringer’s glucose infusion (0.5 mg/kg · hr), which continued throughout the experiment. A servo-controlled heating pad maintained a constant rectal temperature of 38°C.

Pulsatile blood pressure recordings are difficult to obtain from the central ear artery of anesthetized rabbits, and the left femoral artery was therefore cannulated for blood pressure recordings. Blood samples for catecholamine17,18 and metoprolol19 analysis were drawn 10 minutes before (central ear artery) and 90 and 170 minutes after (femoral artery) anesthesia. Additional blood samples were drawn for arterial blood gas analysis. End-expiratory CO₂ was monitored by a CO₂ analyzer (Engström Eliza, Stockholm) connected to the tracheal cannula. The ventilator was adjusted to maintain arterial P0₂ > 10 kPa, P0₂ = 4–5 kPa, and pH (corrected with bicarbonate injections when necessary) 7.4–7.5. Blood pressure and heart rate recordings were displayed on a polygraph (Grass Instrument Co., Quincy, Mass.) via a Statham pressure transducer (Gould Inc., Cleveland) and simultaneously analyzed on a Commodore PC-40 (see below).

The experiments were terminated 240 minutes after the metoprolol/vehicle administration began, that is, 180 minutes after the induction of anesthesia. The rabbits were thoracotomized and perfusion-fixed (pressure, 75 mm Hg) through a coarse needle in the left ventricle and drained through the right atrium. The vascular system was primarily flushed with approximately 700 ml Dulbecco’s solution followed by fixation with 1 l of 10% neutral buffered formalin. All the perfusates had a temperature of 38°C. The thoracic aorta was carefully removed, and care was taken to avoid stretching, bending, and compression of the tissue. The conscious controls were subjected to as little experimental manipulation as possible. They were anesthetized with metohexitol through a marginal vein catheter, and the vascular system was perfusion-fixed and treated as described above.

Immunohistochemistry

After transfer to Petri dishes filled with Tris-phosphate buffered saline (PBS), the aortas were cleaned of fat and excess connective tissue and cut into at least four sections. The most proximal section contained no branching vessel orifices; the remaining sections contained two pairs of intercostal artery orifices each. The sections were cut longitudinally, pinned flat on a square of Teflon with the luminal side up, and incubated with goat anti-rabbit F(αb)₂ IgG fragment (1:100) for 30 minutes, except for the most proximal section, which served as control. After the sections were rinsed (3 × 5 minutes in Tris-PBS), they were incubated with peroxidase-conjugated donkey anti-goat IgG (1:20) for 30 minutes. Both antibodies were purchased from Jackson Immunoresearch Laboratories, West Grove, Pa. After the sections were rinsed, they were developed for peroxidase activity for 5 minutes with 3',5'-diaminobenidine (1 mg/ml), containing 0.01% H₂O₂ for activation. The development was stopped by pouring tap
water over the sections, which were then stored in Tris-PBS for 15 minutes before dehydration.

Häutchen preparations were prepared grossly as described by Schwartz and Benditt. Briefly, the samples were dehydrated in ethanol 35%, 70%, 95%, and 2x100% for 15 minutes in each bath. The endothelial side of the tissue was carefully fixed to nitrocellulose-covered glass slides. The sections were rehydrated in 35% ethanol, and all tissue layers except the endothelial monolayer were peeled away under a dissecting microscope. The nitrocellulose was then dissolved in ether:ethanol (60:40), and the endothelial monolayers were mounted on glass slides with the luminal side up. Thereafter, the nuclei were stained with Mayer's hematoxylin.

**Calculations and Statistical Analysis**

The number of IgG-positive cells in the endothelium was determined in both unbranched and circumostial aortas (Figure 1b) and expressed as a percentage of the total number of cells investigated. We defined circumostial aorta as an area (diameter, 1 mm) surrounding the lumen of a branching intercostal artery of the aorta (Figure 1b). For this purpose, an eyepiece (Graticules Inc.) with a circle and a cross was used. The cross also allowed us to count cells in the low- and high-shear regions separately (Figure 1b). IgG-positive cells and the total number of cells were counted for each of the four branches of the two most proximal pairs of intercostal arteries. The results obtained from one animal were then averaged, and the average value was used in the statistical analysis. A square eyepiece (Graticules Inc.) was used to define the area to be counted in the unbranched aorta. Counting started distal to the first pair of intercostal arteries and was never extended beyond the fourth pair. Care was taken not to include what was defined as circumostial aorta in these measurements. Cell density (cells/field) was determined from the average cell number obtained from four fields. IgG-positive cells were counted until a total number of 100 such cells was found or a minimum of at least 100,000 cells had been investigated. The cells were counted at ×200 magnification.

Aortas from nine rabbits were counted by three observers: two (B.B. and W.B.S.) counted coded specimens, and one person (K.P.) knew from which group the specimens came. Two observers (B.B. and K.P.) used the same microscope (Laborlux, Leitz, Wetzlar, FRG), and the other (W.B.S.) used different equipment (Nikon). The results obtained were compared by linear regression, and good correlation was obtained among the three observers (r=0.91, r=0.99). Therefore, the remaining slides were analyzed blindly by one person (B.B.).

The blood pressure signal was digitalized (200 Hz) for 5 seconds every minute by a microcomputer and averaged over 15-minute periods. For statistical comparisons, data from three 15-minute periods were used, that is, before drug administration (t=0 minutes), just before anesthesia was induced (t=60 minutes), and after 3 hours of anesthesia (t=240 minutes).

All results are presented as mean±SEM. Two-way analysis of variance (ANOVA) was used to test whether the hemodynamic effects caused by metoprolol and/or chloralose treatment were statistically significant. For these comparisons, values obtained at 0, 60, and 240 minutes were used. ANOVA was also used to compare the effects of the chloralose anesthesia on plasma catecholamine levels (values at 60 and 240 minutes) and to compare the differences in endothelial cell death found in the different regions studied in the three groups of rabbits. Student's t test for paired observations was used to test whether there were significant differences in endothelial cell death in the low- and high-shear regions of the circumostial intima at the intercostal artery bifurcations in the three groups studied.

**Results**

Endothelial cell death in the thoracic aorta was estimated in eight conscious controls, eight cloralose-anesthetized rabbits, and seven metoprolol-pre-treated cloralose-anesthetized rabbits (mean plasma metoprolol concentration, 194±26 nM). Blood pressure, heart rate, and arterial catecholamine levels were studied in the last two groups. The conscious controls were left as undisturbed as possible.

Hemodynamic data and arterial concentrations of catecholamines are summarized in Table 1. In conscious rabbits, before metoprolol was given, there were no significant differences in arterial pressure or heart rate between the two groups. The rabbits that were not treated with metoprolol responded to cloralose anesthesia with significant increases in heart rate (p<0.001) and arterial pressure (p<0.001) (Figure 2). These changes were associated with an elevated plasma concentration of norepinephrine (p=0.003), but the plasma concentration of epinephrine was not significantly increased (Table 1).

Compared with unblocked rabbits, the metoprolol-treated rabbits responded to the cloralose anesthesia with markedly lower increase in heart rate (p=0.001) (Figure 2). In the third hour of anesthesia, heart rate was approximately 70 beats/min lower in the β-blocked rabbits than in the unblocked rabbits. After 3 hours of anesthesia, blood pressure was increased approximately 12 mm Hg in the β-blocked rabbits versus 22 mm Hg in the unblocked group (p=0.048) (Figure 2). A significant blood pressure reduction with a slow onset was thus caused by the β-blockade. In the metoprolol-treated group, the plasma concentration of norepinephrine increased during anesthesia, an effect that did not differ significantly from that observed in the unblocked rabbits. These results suggest that the cloralose anesthesia caused sympathetic activation with release of norepinephrine in both these groups. However, the resulting heart rate and blood pressure responses were reduced by metoprolol.
Effects on Endothelial Cell Death

The presence of IgG-positive endothelial cells was estimated in an area remote from the intercostal artery bifurcations (unbranched aorta) and around the bifurcations (circumostial aorta). En face preparations of the endothelial layer from such areas obtained from one conscious control and one chloralose-treated rabbit are shown in Figure 3. Chloralose anesthesia markedly increased endothelial cell death in this example. The effects of anesthesia on endothelial cell death are summarized in Figure 4. In the conscious controls, not subjected to any experimental manipulation, 0.23±0.11% and 1.93±0.40% of the endothelial cells in unbranched and circumostial aortas, respectively, were IgG-positive. A fivefold increase in endothelial cell death was found at both these sites in the chloralose-treated rabbits (p=0.013). Pretreatment with metoprolol inhibited this increase entirely, and compared with the conscious controls, chloralose anesthesia did not affect endothelial cell death in this group. It is of particular interest to compare endothelial cell death in the high- and low-shear areas of the circumostial aorta. Figure 5 shows that a higher endothelial cell death rate was found in the high-shear regions. The difference was statistically significant in the conscious controls and the chloralose-treated rabbits (p=0.009 and p=0.007, respectively), but not in the metoprolol-pretreated rabbits (p=0.399).

Discussion

Atherosclerosis is a focal disease, and the earliest lesions are typically found where the blood vessels are curved or branched. It is suggested that this distribution of the plaques is dependent on the concentration of hemodynamic stresses to these areas.21–23 These areas are also characterized by an increased frequency of injured endothelial cells24,25 and an increased endothelial cell turnover.26 When atherosclerotic lesions develop and grow, an increase in both endothelial turnover27,28 and injury29 is observed. We used the presence of intracellular IgG as a criterion of an irreversibly injured cell. Plasma protein accumulation in injured endothelial cells has been described previously,14 and specific intracellular binding of IgG20 makes this protein suitable as a marker of endothelial cell injury. Using this technique, Hansson and Schwartz15 found an endothelial cell death frequency of 0.19±0.04% in aortas of undisturbed rats, which closely agrees with the 0.23±0.11% found in rabbits in the conscious controls of the present study. Our results showed a marked concentration of endothelial cell death in the circumostial regions in all three groups, with a very high frequency of endothelial cell death in the high-shear region, especially in the chloralose-anesthetized rabbits (Figures 3–5). Recently, a similar distribution of injured cells around ostia was also observed when the effects of psychosocial stress in cynomolgus monkeys were studied (W.B. Straw et al.,

### TABLE 1. Effects on Hemodynamics and Plasma Catecholamine Concentrations of β-Blockade and Chloralose Anesthesia

<table>
<thead>
<tr>
<th></th>
<th>Chloralose (n=8)</th>
<th>Chloralose+metoprol (n=7)</th>
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<tr>
<td></td>
<td>0 min</td>
<td>60 min</td>
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<td>Blood pressure (mm Hg)</td>
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<tr>
<td></td>
<td>80±4</td>
<td>79±3</td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>234±9</td>
<td>236±14</td>
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<tr>
<td>Norepinephrine (nM)</td>
<td>. . .</td>
<td>1.28±0.30</td>
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<tr>
<td>Epinephrine (nM)</td>
<td>. . .</td>
<td>0.16±0.06</td>
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The hemodynamic and plasma catecholamine data used in the statistical analysis, that is, values obtained before drug treatment (basal values, t=0), after 1 hour of metoprolol/saline treatment (t=60 minutes), and after 3 hours of chloralose anesthesia (t=240 minutes). As stated in column headings, n=8 and 7, except as indicated in parentheses. Epinephrine concentrations were often below the detection limit.
unpublished data). This pattern corresponds with the predilection sites of atherosclerosis development in cholesterol-fed rabbits.29,31

To establish a link between sympathetic activation and endothelial integrity in these acute experiments, we used 3 hours of chloralose anesthesia as a model of sympathetic activation. Reported not to hamper reflex activation of the sympathetic nervous system,32 chloralose significantly increased blood pressure, heart rate, and plasma catecholamine levels (especially norepinephrine) in our experiments. The effects were stable throughout the 3-hour period of anesthesia and were very reproducible. These results show the relevance of using chloralose treatment to increase the sympathetic activity. The anesthesia also increased the sympathetic activity in the $\beta$-blocked
rabbits, shown by a similar increase in plasma nor-
epinephrine as in the unblocked rabbits (Table 1). However, the hemodynamic effects of the anesthesia were significantly affected by the pretreatment with the β-blocker, especially the heart rate response, which was markedly reduced. Blood pressure was lower in the β-blocked rabbits only toward the end of the experiments; this finding is consistent with the delayed hypotensive response to β-blockade described previously.33 In this model, we observed that sympathetic activation increased endothelial injury and that this effect could be inhibited by metoprolol in plasma concentrations that were in "the therapeutic range."34 The effect of β-blockade supports the conclusion that the effects of chloralose anesthesia on endothelial integrity resulted from an increase in sympathetic activity, mediated via β-adrenoceptor activation. Stressful stimuli have previously been shown to cause intimal lesions,35 and metoprolol was recently observed to decrease endothelial injury in monkeys subjected to psychosocial stress (W.B. Strawn et al, unpublished observations). Intimal lesions can also be caused by direct electrical stimulation of the hypothalamus.36 The present findings directly demonstrated that such effects might be mediated via sympathetic activation and β-adrenoceptor stimulation and thus provide a possible explanation for the antiatherosclerotic effects of β-blockers previously described.6,7 The effects of sympathetic activation found were paralleled by effects on blood pressure and heart rate. In view of the hypothesis concerning a relation among hemody-
namic factors, endothelial injury, and atherosclerosis (see above), it is tempting to speculate that the inhibitive effect of β-blockade on the development of endothelial cell injury found in the present study was also mediated by hemodynamic factors. Consequently, the antiatherogenic effect of β-blockers might also be mediated by the hemodynamic effects of the drug. The specific hemodynamic factor that might accelerate atherogenesis is unknown. In cholesterol-fed rabbits, plaque development is normally associated with high-shear stress,28,29 whereas plaque formation in, for example, the human carotid bifurcation is associated with low-shear stress.23 High heart rate has been suggested as a risk factor for atherosclerosis,37 and a reduction in wall stress (from blood pressure) and the oscillatory movements of the vessel wall caused by the action of the heart has been suggested to reduce atherosclerosis development.38 The link between hemodynamics and atherogenesis is thus complex, and our results do not allow us to definitely conclude that our findings on endothelial integrity were mediated via hemodynamic factors. There are in fact many other possible mechanisms by which the sympathetic nervous system and β-blockers can exert their effects on atherogenesis (see References 39 and 40).

There are some clinical implications of the present findings. In hypertensive patients, metoprolol decreased cardiovascular morbidity and mortality compared with thiazide diuretics, even though the
two agents caused similar blood pressure reduction. In the treatment of patients after myocardial infarction, metoprolol reduces not only mortality and the incidence of reinfarction but also the incidence of stroke and peripheral arterial disease. These findings suggest that metoprolol reduces either atherosclerosis or related arteriothrombotic events. Because the intact endothelium is nonthrombogenic and the injured endothelium is a focus for platelet adhesion and aggregation, the present data may be taken to suggest that the effects observed in patients might be mediated via a reduction of endothelial injury, as found in this study.

In conclusion, this study showed that aortic endothelial cell death was increased in a model of sympatheticoactivation and that this effect was prevented by pretreatment with the β1-blocker metoprolol. Endothelial cell death was markedly higher at arterial branching points than in unbranched aorta. These findings may explain the accelerated development of atherosclerosis associated with psychosocial stress, and they also provide evidence to explain the antiatherosclerotic effects of β-adrenoceptor antagonists.

References

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**KEY WORDS** • sympathetic activation • endothelial injury • β₁-adrenoceptors • β-antagonists • rabbits
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