Endothelial Dysfunction in Response to Psychosocial Stress in Monkeys

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The current study was designed to evaluate the effects of a disrupted social environment on the endothelial integrity of various vascular segments in male cynomolgus monkeys (Macaca fascicularis). Each of 20 single-caged adult monkeys was fed a diet comparable to a person’s ingestion of 240 mg cholesterol/day for a 10-week baseline period and then was introduced as a stranger into a four-member social group for 3 days. Half of the monkeys received a β-adrenergic blocking agent (metoprolol) via subcutaneous implant 2 days before and during group housing. The social manipulation produced persistent sympathetic arousal as evidenced by significantly elevated heart rates among untreated monkeys (p<0.01) but not among their metoprolol-treated counterparts, whose heart rate declined (p<0.05). After the social manipulation, all monkeys were necropsied and evaluated for endothelial incorporation of immunoglobulin G (as an indicator of cell death), endothelial cell replication, the presence of adherent leukocytes, and arterial low density lipoprotein permeability and concentration. At branching sites in the thoracic aorta, immunoglobulin G incorporation and endothelial cell replication were significantly greater in untreated monkeys than in metoprolol-treated monkeys (p<0.01 for both analyses); no differences existed at nonbranch sites. Endothelial cell replication in the coronary arteries (where immunoglobulin G incorporation was not examined) was also greater among untreated than among metoprolol-treated monkeys. No significant differences were observed between treatment groups in arterial low density lipoprotein permeability or leukocyte adherence; estimates of arterial low density lipoprotein concentrations were higher among untreated than among metoprolol-treated monkeys, but only in the abdominal portion of the aorta. These results indicate that social disruption is associated with both sympathetic nervous system arousal and indexes of endothelial dysfunction, effects that may be prevented by treatment with a β-adrenergic blocking agent. (Circulation Research 1991;68:1270–1279)

There is increasing evidence that psychosocial factors contribute to the development of atherosclerosis. In experimental studies of cholesterol-fed cynomolgus monkeys (Macaca fascicularis), for instance, we have previously demonstrated that chronic exposure to stress (repeated disruption of social group memberships) potentiates coronary artery atherogenesis among animals of high social rank (i.e., socially dominant monkeys). The behavioral exacerbation of atherosclerosis in this model has two features: 1) it cannot be attributed to concomitant variability in the animals’ serum lipid concentrations, and 2) it is mitigated by administration of a β-adrenergic blocking agent, propranolol HCl. We have also observed that monkeys that exhibit the largest heart rate (HR) responses to a standardized behavioral challenge (threat of capture) have more extensive coronary artery atherosclerosis on necropsy than monkeys that show a less pronounced cardiac responsivity under the same stimulus conditions. Together, these findings suggest that behavioral influences on atherosclerosis are largely mediated by activation of the sympathetic nervous system, possibly via effects associated with the animals’ acute hemodynamic reactions to stress. With respect to the potential atherogenicity of HR itself, Beere et al have reported that cynomolgus monkeys having “low” HRs—as defined by natural variability (animals at the lower end of the HR distri-
bution) or achieved experimentally by sinoatrial node ablation—developed only half the coronary atherosclerosis observed in monkeys with more elevated HRs, after 6 months of consumption of a cholesterol-containing diet. This effect also could not be accounted for by corresponding group differences in serum lipid concentrations, blood pressure, or body weight. Hence, Beere et al.6 conclude that elevated HR may represent a predisposing hemodynamic factor in atherogenesis and (consistent with our own observations) speculate that psychosocial influences on coronary artery disease may be mediated by persistent elevations in HR and associated flow disturbances (e.g., turbulence and shear stress).

When in the natural history of atherosclerosis such perturbations might influence lesion development most appreciably is unclear, but it might be suggested that hemodynamic factors, if important, contribute in some measure to early endothelial damage.1 In this regard, there is considerable evidence that the beginnings of the atherosclerotic process occur when some form of “injury” is sustained by the arterial endothelium.2,8 Whereas the normal endothelium exhibits limited permeability to macromolecules, such as lipoproteins, and serves as a thromboreistant barrier, the sequelae of endothelial damage include 1) an intimal accumulation of lipoproteins; 2) adherence of platelets; 3) the release of mitogenic substances by regenerating endothelial cells, platelets, or adherent leukocytes; and 4) intimal smooth muscle cell proliferation.9,10 There is also some evidence that psychosocial distress alone can induce endothelial injury. This is demonstrated in experimental studies showing common laboratory stressors (e.g., electric shock and physical restraint) to induce a rapid turnover of endothelial cells in the rat aorta, accompanied by subendothelial accumulation of mononuclear leukocytes and morphological changes in the arterial intima.11,12

Recently, we observed that rabbits subjected to chloralose anesthesia leading to adrenergic activation displayed an increase in aortic endothelial injury.13 Chloralose anesthesia was accompanied by increases in HR and blood pressure, suggesting β1-adrenoceptor effects of the treatment. When a selective β1-adrenoceptor antagonist was given to the rabbits, both the increase in HR and the increase in endothelial injury could be inhibited, supporting the concept of a cause–effect relation between adrenergic activation, HR, and endothelial injury.

The effects of stress on aortic endothelial injury in nonhuman primates (a more representative model of human atherosclerosis) have not previously been evaluated. Likewise, the effects of stress on the endothelium of coronary arteries have not been studied. Accordingly, in the present investigation we evaluated the effects of a disrupted social environment on the endothelial integrity of different vascular segments, including coronary arteries, of male cynomolgus monkeys. Inasmuch as unmanipulated animals also may have increased and variable degrees of adrenergic activation, we decided to use monkeys subjected to the same psychosocial stressor, but under the protection of a β1-adrenoceptor blocking agent, as treated controls. The principal dependent measures included nondenuding endothelial injury (evaluated with immunoglobulin G [IgG] immunohistochemistry).14,15 Endothelial cell turnover (as an independent, albeit indirect, measure of injury), leukocyte adherence to the arterial surface, and lipoprotein interactions with artery.

Materials and Methods

Animals and Diet

Twenty adult male cynomolagus macaques (Macaca fascicularis) weighing 5.19±0.20 (mean±SEM) kg and recently imported from Indonesia were studied. This species was selected because of its susceptibility to atherosclerosis and its demonstrated usefulness in studies evaluating behavioral influences on coronary atherosclerosis.16 The 10 weeks before the behavioral and pharmacological manipulations comprised a “baseline” period. During the baseline period, the monkeys were fed a diet that derived 43% of calories from fat and contained 0.10 mg cholesterol/calorie. The purpose of feeding this diet was not to induce atherosclerosis but rather to mimic the dietary intake of human beings; in contrast, the usual laboratory monkey diet (“monkey chow”) differs markedly from diets typically consumed by human populations. The experimental diet produced total plasma cholesterol (TPC) concentrations typical of those observed among North American humans who would not be considered at high risk for developing coronary heart disease (mean TPC=189 mg/dl).

Experimental Design

Social manipulation. We have previously demonstrated that the social disruption occasioned by exposure to new social groupings and social strangers potentiates atherogenesis, even among monkeys not consuming a diet high in saturated fat and cholesterol.17 We18 also observed previously that monkeys display elevated HR on exposure to such disruption, suggesting that a monkey’s physiological response to the challenge of a new social setting is characterized by sympathetic nervous system arousal. In the current investigation, we used a modified social disruption manipulation, designed to acutely challenge specific “target” monkeys and elevate their HRs. The protocol involved exposure of each experimental monkey, individually, to a psychosocial stressor. Specifically, after the 10-week baseline period, during which all experimental monkeys were housed in individual cages, social disruption was accomplished by placing an experimental (target) monkey together with four “host” monkeys (randomly chosen from a pool of five nonexperimental monkeys, all social strangers to the target monkey) for a period of 3 days. At the end of this time, the target monkey was removed from the group, the experiment was terminated, and all host monkeys were returned to indi-
vidual cages. This procedure was repeated each week with four randomly chosen host monkeys and a new target monkey until all 20 experimental monkeys were manipulated as targets. HRs and plasma lipid concentrations were measured in target monkeys during the baseline and experimental periods.

Pharmacological manipulation. The 20 experimental monkeys were assigned in equal numbers to either metoprolol-treated or untreated groups. In both groups, a subcutaneous osmotic minipump (model 2ML1, Alza Corp., Palo Alto, Calif.) was implanted 3 days before the beginning of the social manipulation. The pump delivered 15 mg metoprolol/kg/day to the treated group from the time of implantation until necropsy; the untreated group received saline.

In Vivo Determinations

Plasma lipid concentrations and body weight. Blood samples for determination of TPC and high density lipoprotein (HDL) cholesterol concentrations were obtained, after an 18-hour fast, two times during the baseline period and once again after the experimental manipulation, just before necropsy. Measurement of TPC was accomplished by enzymatic methods; plasma HDL cholesterol concentrations were determined by the heparin manganese precipitation procedure. Plasmas low density lipoprotein (LDL) cholesterol concentration was not measured directly; rather, it was estimated as the difference between HDL cholesterol and TPC. This approximation of LDL cholesterol was considered reasonable in view of the low triglyceride values typically observed in this species (<25 mg/dl), suggesting low values for very low density lipoprotein (VLDL). In addition, for six of the experimental monkeys, cholesterol in the VLDL fraction (<1.006 g/ml) was measured and corrected for ultracentrifugal loss. The mean value for VLDL cholesterol was 3.40±0.93 mg/dl, which was less than 4% of non-HDL cholesterol. Therefore, we assumed that non-HDL cholesterol was equal to LDL cholesterol. Body weight was measured at the same time blood samples were taken for serum lipid determinations.

Heart rate measurements. All HR recordings were via radiotelemetry; in this procedure, portable electrocardiographic transmitter units (LSE Inc., Tullahoma, Tenn.) were attached to monkeys with electrodes and secured beneath nylon mesh jackets. Recordings of HR were obtained in the afternoon on three occasions during the baseline period and at the same time of day during each of the 3 days of the social manipulation. All monitoring of HR was accomplished while the investigator was positioned in a separate room from the monkeys. On each occasion, HR was sampled over a 15-minute period; during these times, the electrocardiograph was charted continuously. Quantification of HR was achieved by summing all R waves detected within each sample: mean baseline HR values (expressed in beats per minute) were computed as an average of the recordings made during each of the three baseline record-

ing sessions, and mean experimental HRs were computed similarly from the three experimental period recording sessions.

Postmortem Determinations

Tissue preparation. At the end of the experiment, all monkeys were anesthetized with ketamine (30 mg/kg i.m.) and then injected with sodium pentobarbital (1 mg/kg i.v.). While deeply anesthetized, monkeys were exsanguinated, flushed with phosphate buffer to remove residual blood containing radiolabeled lipoproteins (pH 7.4), and pressure-perfused at 100 mm Hg with 4% normal buffered formalin. After fixation, the thoracic aorta was placed in phosphate buffer before immunocytochemical staining and subsequent autoradiography of en face (Hautchen) preparations of the endothelium. The abdominal aorta, the basilar and vertebral arteries, the right and left common carotids and their bifurcations, and the left anterior descending and the left circumflex coronary arteries were placed in modified Karnovsky's fixative. Fixation in Karnovsky's solution preserves [125]Ityramine cellobiose ([125]ITC) present on undegraded LDL and nearly all of the [125]ITC present on products of arterial LDL degradation, but only the [125]I bound to undegraded LDL. After fixation for 24 hours, arterial samples were prepared for light microscopy, gamma counting, or scanning electron autoradiography.

Intimal thickness measurement. Thickness of arterial intima was evaluated, because all monkeys consumed a cholesterol-containing diet. Intimal thickness was measured in the carotid and coronary arteries from standard paraffin-embedded sections after staining with Verhoeff–Van Gieson stain using an image analyzer (Morphometer, WHEA, Woods Hole, Mass.).

Identification of injured endothelial cells. Irreversibly injured endothelial cells were identified by combining immunohistological staining of autologous IgG and Hautchen preparation of the thoracic aorta endothelium, as previously described. In brief, after fixation, the endothelial surface of the thoracic aorta was exposed and incubated with F(ab')2 fragment goat anti-monkey IgG antibody (8.0 mg protein/ml, 1:1,000 dilution, Cappel, West Chester, Pa.), followed by biotinylated rabbit anti-goat antibody (1:500 dilution, Vector Laboratories, Inc., Burlingame, Calif.) and peroxidase-conjugated streptavidin-biotin (1:500 dilution, Amersham Corp., Arlington Heights, III.). Peroxidase was visualized with 0.02% hydrogen peroxide added to an equal volume of 0.1% dianaminobenzidine tetrachloride made in 0.1 M Tris buffer (pH 7.2). Antibodies were used at optimal concentrations, determined by checkerboard titrations on spleen sections and Hautchen preparations of the aorta. IgG-positive (IgG+) cells were counted through a calibrated eyepiece grid at ×400 magnification and were expressed as a percentage of the total number of endothelial cells counted. Sites associated with both the intercostal artery branches and the non-
branched aorta were investigated. Branch-associated aorta was defined as the area 0.5 mm on either side of and 2 mm proximal and distal to the center of each intercostal ostial space; only the four most proximal pairs of intercostal artery branches were described. Nonbranched aorta was defined as within the area beginning 2 mm distal to the first pair of intercostal ostia, ending 2 mm before reaching the fourth pair of ostia, and excluding the intervening ostial sites. At least 50 IgG+ cells were identified, and at least 100,000 endothelial cells were counted per aorta.

Endothelial cell replication. The presence of endothelial nuclei in S phase can be identified by \(^{3}H\)thymidine \((^{3}HT)\) incorporation. We used endothelial cell replication (ECR) as an indirect index of endothelial cell damage for two reasons: 1) replicating endothelial cells occur at very low frequencies in the adult animal unless stimulated to replicate, for example, by endothelial injury, and 2) cell densities were consistent in these animals, at least as evaluated in the thoracic portion of the aorta. To assess ECR, autoradiography was used with both scanning electron autoradiographic preparations (in the left circumflex and left anterior descending coronary arteries) and the Hautchen preparations (in the thoracic aorta), described above to identify \(^{3}HT\) incorporation into replicating endothelial cell nuclei.

Briefly, \(^{3}HT\) (1.5 mCi/kg total, New England Nuclear, Boston) was administered in three equal doses by intramuscular injection to each monkey at 17 hours, 9 hours, and 1 hour before necropsy. Tissues were prepared for scanning electron autoradiography as previously described. Two hundred fields (0.006 mm\(^2\)/field) per sample were examined for \(^{3}HT\)-positive \((^{3}HT+)\) endothelial cells at a magnification of \(\times1,250\). Scanning electron autoradiography was performed on all tissues except those from the thoracic aorta. The thoracic aorta Hautchen preparations were prepared for autoradiography as described elsewhere. \(^{3}HT\)+ endothelial cells were quantified in the same manner and in the same tissue as the IgG+ cells.

Adherent leukocytes. Adherent leukocytes, often associated with cell injury, were also quantitated by scanning electron autoradiography concurrently with \(^{3}HT\)+ endothelial cells. Such leukocytes were easily distinguishable from endothelial cells by their size (8–15 \(\mu\)m), round shape, and raised appearance above the surface of the tissue.

Preparation and labeling of LDL. Six nonstudy cynomolgus monkeys fed the same diet as the target monkeys served as donors for plasma from which LDL was isolated for these experiments. Plasma was obtained from blood collected from these donor monkeys into disodium EDTA at a final concentration of 1 mg/ml LDL (density, 1.020–1.063 g/ml) was prepared by differential centrifugation, followed by exhaustive dialysis against buffer (0.9% NaCl, 0.01% EDTA, pH 7.4). After dialysis, LDL protein was determined with bovine serum albumin as a standard. The radiolabels for the LDL were obtained from Amersham. One aliquot of each LDL preparation (10–15 mg protein) was iodinated directly with \(^{125}I\) while a second aliquot (20–30 mg protein) was coupled to \(^{125}I\) at 0.1 \(\mu\)mol TC/10 mg LDL protein. Less than one molecule of TC was bound per molecule of apoprotein B. The specific activities ranged from 150 to 2,400 cpm/ng protein for \(^{131}I\), and from 50 to 900 cpm/ng protein for \(^{125}I\). The LDL preparations were analyzed by electrophoresis on 4–22% gradient polyacrylamide gels after denaturation with sodium dodecyl sulfate and on agarose gels by precipitation with trichloroacetic acid and by extraction with chloroform/methanol. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that more than 95% of both radiolabels were associated with apoprotein B. Agarose gel electrophoresis showed that more than 95% of the radioactivity was present in a single peak with \(\beta\)-mobility. Less than 5% of the radioactivity could be extracted into 1:1 chloroform/methanol. Less than 4% of the radioactivity was soluble in 10% final concentration of trichloroacetic acid.

Arterial permeability to LDL. The arterial permeability to LDL was measured using monkey LDL coupled to \(^{125}I\) as described above. Accordingly, monkeys were injected with \(^{125}I\)-monkey LDL (0.250±0.001 [mean±SEM] \times 10^8 cpm/kg). For measurement of plasma radioactivity, blood samples were collected at 5, 10, 15, and 30 minutes after injection and just before termination of the experiment, 1 hour after injection. Because \(^{125}I\) remains within cells after cellular degradation of \(^{125}I\)-LDL, use of the TC label ensures that our measure of arterial permeability to LDL was not diminished by cellular degradation of LDL during the experiment. In addition, after a period of arterial uptake of LDL as short as the 1 hour used here, efflux of labeled LDL from the artery should be minimal, allowing the accurate calculation of arterial permeability to LDL. The arterial permeability to LDL was calculated as 

\[
k = \frac{a}{AUC_{\text{plasma}}}
\]

where \(k\) is arterial permeability to LDL (\(\mu\)l/hr/cm\(^2\)), \(a\) is the arterial content of \(^{125}I\) label at the end of the experiment (cpm/cm\(^2\)), and \(AUC_{\text{plasma}}\) is the area under the plasma radioactivity curve (cpm \cdot hr/\(\mu\)l) from injection until the end of the experiment.}

Estimate of the arterial concentration of undegraded LDL. In this study, we estimated the arterial concentration of undegraded LDL using data obtained after injection of \(^{131}I\)-LDL (1.58±0.23 [mean±SEM] \times 10^8 cpm/kg). Blood samples were collected during the first hour after injection and during the last hour before termination of the experiment, 2 days after injection. To estimate the arterial concentration of undegraded LDL, we assumed that all the undegraded LDL in artery equilibrates with the plasma LDL. This assumption is probably reasonable for the arteries of these monkeys, which were free from intimal thickening (see below). Given this assump-
tion and the prolonged (2-day) period of arterial exposure to labeled LDL, the arterial concentration of undegraded LDL can be estimated in terms of a percentage of the plasma LDL concentrations: estimated arterial concentration of undegraded LDL = 100 C_t/C_p where C_t is the arterial content of protein-bound radioactivity and C_p is the protein-bound radioactivity in plasma at death.36,37

Provided that LDL in artery exchanges freely with LDL in plasma, this ratio accurately reflects the mass ratio only at the instant when the curve of radioactivity in the artery peaks, which has been reported to be between 4 and 8 hours after injection of LDL into normal rabbits.38,39 Afterwards, this ratio overestimates the arterial LDL concentration, but probably by 10–15% or less.35,36 We estimated the arterial content of LDL in absolute terms by multiplying the ratio C_t/C_p by the plasma LDL cholesterol concentration (µg/ml plasma).

### Statistical Analysis

Analyses of variance (ANOVAs) or analyses of covariance (ANCOVAs) were used for all comparisons. Where appropriate, a square-root transformation (x' = √x + √x + 1) was used, as recommended by Sokal and Rohlf40 to yield distributions suitable for parametric analysis. All tests of significance were two-tailed.

### Results

**Antemortem Determinations**

Evaluation of all antemortem data was accomplished with the use of 2x2 repeated-measures ANOVAs (treatment = untreated, metoprolol × period = baseline, experimental).

### Plasma lipids

Plasma lipids. Plasma lipid concentrations as measured during the baseline period and again at the end of the experimental manipulation are shown in Table 1. Analysis revealed no significant effects of treatment and no interaction between treatment and period for either TPC or HDL cholesterol concentrations; however, there was a significant main effect of period, reflecting a drop in both TPC and HDL cholesterol concentrations between the baseline and experimental periods (p < 0.001 for both). Analysis of the ratio of TPC to HDL cholesterol revealed no significant effects.

**Body weight.** The body weights of monkeys measured during the baseline period and after the experimental manipulation are shown in Table 2. The ANOVA performed on body weight measurements revealed no effects of treatment and no interaction of the treatment and period factors. Like plasma lipid concentrations, however, there was a significant main effect of period, which reflected a loss of weight associated with the experimental manipulation (relative to baseline) across all monkeys.

**Heart rate.** The HR data are shown in Table 2. Analysis of these data revealed no main effects for either treatment or period. There was, however, a significant interaction between these factors (p < 0.001), reflecting a substantial increase in HR among the untreated monkeys and a decline among the metoprolol-treated monkeys, associated with the experimental manipulation. Post hoc testing by Scheffe's technique revealed no significant difference between the HRs of metoprolol-treated and untreated monkeys during the baseline period. On the other

### Table 1. Mean Plasma Lipid Concentrations During the Baseline Period and After Experimental Manipulation in Untreated and Metoprolol-Treated Monkeys

<table>
<thead>
<tr>
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<th>Untreated (n=10)</th>
<th>Metoprolol (n=10)</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Postexperimental</td>
</tr>
<tr>
<td>TPC (mg/dl)</td>
<td>191±35</td>
<td>150±41*</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>71±21</td>
<td>54±18*</td>
</tr>
<tr>
<td>TPC/HDL</td>
<td>2.94±1.0</td>
<td>3.14±1.78‡</td>
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</tbody>
</table>

Values are mean±SD and represent the average of two samples from the baseline period and one sample after the experimental manipulation. TPC, total plasma cholesterol; HDL, high density lipoprotein cholesterol.

*p < 0.01 vs. within-group baseline value by analysis of variance (ANOVA).

†p > 0.20 vs. corresponding value for the untreated group by ANOVA.

‡p > 0.20 vs. within-group baseline value by ANOVA.

### Table 2. Mean Body Weight and Heart Rate asMeasured During the Baseline Period and After the Experimental Manipulation in Untreated and Metoprolol-Treated Monkeys

<table>
<thead>
<tr>
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<th>Untreated (n=10)</th>
<th>Metoprolol (n=10)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Postexperimental</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>5.18±0.70</td>
<td>4.86±0.66*</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>126±19</td>
<td>164±23‡</td>
</tr>
</tbody>
</table>

Values are mean±SD. Body weight from the baseline period represents the average of two samples; a single weighing was performed after the experiment.

*p < 0.001 vs. within-group baseline values by analysis of variance (ANOVA).

†p > 0.20 vs. corresponding value for untreated group by ANOVA.

‡p > 0.20 vs. within-group baseline value by ANOVA.

§p < 0.001 vs. postexperimental value for treated group by ANOVA.
hand, there was a significant (42 beat/min) difference between groups during the manipulation ($p<0.05$). Nonetheless, despite randomization, the baseline HRs of the metoprolol-treated monkeys were 20 beats/min greater than those of the untreated monkeys; because we expected HR to influence the experimental outcome, baseline HR thus was used as a covariate in all analyses of data collected postmortem.

**Postmortem Determinations**

All evaluations of postmortem data were accomplished by means of one-factor ANCOVAs (treatment, untreated, metoprolol); as stated above, HR recorded during the baseline period was used as the covariate.

**Intimal thickness.** Table 3 contains the data describing intimal thickness in the coronary and common carotid arteries and in the abdominal portion of the aorta. The ANCOVA applied to these data revealed no significant difference between groups at any site. Further, intimal thickness in all cases was consistent with the absence of atherosclerosis (i.e., <0.10 mm).

**Endothelial dysfunction.** Endothelial injury was evaluated both directly, with the IgG immunoperoxidase technique, and indirectly, through ECR rates. Importantly, data from the thoracic aorta indicated that an increased ECR was correlated with an increased frequency of injured endothelial cells ($r=0.48, p<0.05$). Therefore, we concluded that variations in ECR also reflected endothelial injury.

Data describing the frequency of injured endothelial cells in thoracic aorta are depicted in Figure 1. The two groupings in the figure represent data from branched and nonbranched sites respectively. An ANCOVA applied to these data reveals a significant effect associated with treatment, with lower frequencies of injured endothelial cells in metoprolol-treated than in untreated monkeys, but only at the branching sites ($p<0.001$). Untreated and metoprolol-treated monkeys did not differ in the frequency of IgG+ cells at nonbranched sites.

Data reflecting ECR in the thoracic aorta are depicted in Figure 2. Results of ANCOVAs applied to these data indicated significantly less ECR among metoprolol-treated monkeys, but again, only at branching sites ($p<0.02$); there were no differences in ECR between untreated and metoprolol-treated monkeys in nonbranched areas.

The ECR data representing the mean values for the coronary arteries (left circumflex and left anterior descending combined) are shown in Figure 3. Analysis of these data (by ANCOVAs) revealed the ECR to be significantly lower in the endothelium of metoprolol-treated as compared with untreated monkeys ($p<0.02$). Although these data, as transformed, satisfy the conditions for parametric evaluation, the counts of replicating cells were relatively low. Thus, an additional, nonparametric procedure was applied to these data. Specifically, ECR was compared with a Mann-Whitney $U$ test in the six pairs of metoprolol-treated and untreated monkeys that could be matched for baseline HR. This comparison yielded results similar to those from the ANCOVA. That is,
ECR was significantly lower in treated monkeys than in their untreated counterparts (medians: 0.0 for treated, 0.625 for untreated, p<0.05).

Adherent cells. The data describing the number of adherent cells observed at each of the arterial sites is contained in Table 4. Analyses by ANCOVA revealed no differences between the untreated and metoprolol-treated monkeys at any arterial site.

LDL permeability and estimated arterial LDL concentrations. Values reflecting LDL permeability and estimates of arterial LDL concentration for each arterial site are shown in Table 5. There was a trend toward a difference in LDL permeability between untreated and metoprolol-treated monkeys in the carotid artery (p<0.10), but at no other sites. Estimated concentrations of LDL freely exchanging with plasma LDL were significantly greater within the abdominal portion of the aorta of control monkeys than among their metoprolol-treated counterparts (p<0.02); estimates of LDL concentrations were equivalent among control and metoprolol-treated monkeys at all other sites.

Discussion

The major observation of the present study is that monkeys subjected to an acute psychosocial stressor have evidence of endothelial injury when compared with monkeys with β1-adrenoceptor blockade. This observation was validated directly, with measures of injured endothelial cells (i.e., incorporation of IgG), and indirectly, through endothelial replication rates. The observation is valid for the coronary arteries, as well as the aorta, with the effects in coronary arteries of particular interest because of their potential relevance to coronary artery atherogenesis among humans. The endothelial effects described above were paralleled by stress-associated differences in the HRs of untreated and metoprolol-treated monkeys, differences that did not exist in the baseline period. The marked elevation of HR during the experimental period among untreated monkeys and the concomitant attenuation of HR among monkeys treated with metoprolol support two conclusions: 1) the introduction of monkeys to a strange social environment triggered a significant, persistent cardiac response; and 2) this cardiac response was probably mediated by activation of the sympathetic nervous system.

It has been observed previously in this species that variability in HR is associated with atherosclerosis. Hence, low HRs (naturally occurring or surgically induced) are associated with a significant inhibition of diet-induced atherosclerosis.5 Our own findings have demonstrated an inhibition of atherosclerosis among behaviorally predisposed monkeys in stressful social situations following chronic treatment with a β-adrenergic blocking agent (propranolol). Conversely, our studies5,4,1 have shown that both elevations in “casual” HRs of monkeys living in stressful social situations and heightened HR responses to psychological challenge are associated with exacerbation of diet-induced atherosclerosis. In all of these studies, concomitant variability in serum lipids or other risk factors cannot account for the behavioral exacerbation of atherosclerosis. Furthermore, principal effects on lesion development were limited to sites presumed to be most vulnerable to damage induced by hemodynamic factors (e.g., the coronary arteries).

The data from the current experiment bridge the observations from previous studies of rodents, rabbits, and monkeys and lead us to speculate that sympathetically induced elevations in HR can initiate early events in atherogenesis (i.e., nondenuding endothelial injury). The association of elevated HRs with psychosocial stress suggests, in turn, that the influence of psychosocial factors on atherosclerosis and coronary heart disease is mediated, at least in part, by the hemodynamic concomitants of sympathetic arousal.

The observation that direct measures of the frequency of injured endothelial cells and endothelial

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

**Figure 3.** Bar graph showing the number of endothelial cells per square millimeter (mean±SEM) with evidence of replication in the coronary arteries in monkeys. (Data are averaged from the left anterior descending and left circumflex arteries.)

### Table 4. Adherent Cells at Arterial Sites in Untreated and Metoprolol-Treated Monkeys

<table>
<thead>
<tr>
<th>Site</th>
<th>Adherent cells (number/mm²)</th>
<th>Untreated</th>
<th>Metoprolol</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary artery</td>
<td></td>
<td>2.21±1.91</td>
<td>2.79±2.40</td>
<td>NS</td>
</tr>
<tr>
<td>Left common carotid artery</td>
<td></td>
<td>1.67±1.47</td>
<td>3.04±2.52</td>
<td>NS</td>
</tr>
<tr>
<td>Left carotid bifurcation</td>
<td></td>
<td>2.24±1.41</td>
<td>3.42±3.4</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean±SD. NS, not significant.

*pOne-factor analysis of covariance was used to evaluate the effects of treatment (untreated vs. metoprolol-treated groups), with heart rate in the pre-experimental period as a covariate.
cell replication rates give similar information may be of relevance for future studies in monkeys, since the IgG immunoperoxidase technique has not previously been applied to nonhuman primates. Additionally, the present data lend strong support to the concept that endothelial cell replication rates reflect endothelial injury in the present model, rather than growth or changes in cell density.

Effects of adrenergic activation and blockade on serum lipoprotein levels have been a subject of considerable interest within cardiovascular medicine.\textsuperscript{42-44} In the present study, we observed decreases in both TPC and HDL cholesterol. A decrease in HDL induced by \( \beta \)-blockade has been of concern to many clinicians in view of the significance of HDL as a protective factor for coronary heart disease. The present data do not support the view of a negative effect of \( \beta \)-blockade on HDL, because HDL was comparable in metoprolol-treated and untreated monkeys. On the other hand, we have no explanation for the parallel decreases in both TPC and HDL cholesterol associated with the psychosocial stress manipulation. However, as body weight also decreased during the experimental stress period, it might be speculated that effects of the stressor on food intake were of significance.

Of other observations, it might be pointed out that our estimates for LDL concentrations were significantly lower in the abdominal aorta of metoprolol-treated monkeys than in corresponding regions of untreated monkeys. However, no other differences were observed regarding estimated LDL concentrations between treated and untreated monkeys. Likewise, the treatment did not affect LDL permeability significantly. And finally, no differences between treated and untreated monkeys regarding leukocyte adhesion were observed.

In the dominant paradigm concerning the pathogenesis of atherosclerosis, endothelial injury, leukocyte adhesion, and LDL deposition in arterial tissue are all regarded as early indicators of the disease. Therefore, it may seem anomalous that only endothelial injury is clearly affected by the present, presumably atherogenic experimental manipulation. This apparent anomaly may be due to differences in the degree of variation between animals for different physiological processes, the selection of arterial sites in which to measure these indexes of atherosclerosis, or the time sequence of the pathogenesis. For example, low numbers of leukocytes adhering to the arterial surface may lead to large variability, thus obscuring actual differences. An argument against this reasoning would be that leukocyte counts actually are almost identical between the metoprolol-treated and untreated groups. Alternatively, the lack of difference between the groups may reflect the fact that leukocytes were counted only at the surface and, therefore, the numbers of subendothelial leukocytes might still be different. The data of Gordon et al\textsuperscript{11} from rats subjected to laboratory stress, indicating an increased number of subendothelial leukocytes, would support this possibility. Additionally, it is possible that the 3-day experimental period was not sufficient for the manifestation of leukocyte effects. With respect to arterial permeability to LDL or estimates of arterial concentrations of undegraded LDL, an absence of a treatment effect may be due to the difficulty of detecting small differences when underlying biological variation is large. Alternatively, the absence of an effect of treatment on LDL interaction with artery may reflect different responses of various arterial sites to metoprolol treatment. Hence, in the coronary artery (an arterial site that is highly susceptible to exacerbation of atherosclerosis by psychosocial stress), the 63% decrease in permeability to LDL, conferred by metoprolol treatment, although not statistically significant, was similar in magnitude to the decrease in endothelial cell replication rate at

\begin{table}
\centering
\caption{Low Density Lipoprotein Permeability and Estimated Concentration at Various Arterial Sites in Untreated and Metoprolol-Treated Monkeys}
\begin{tabular}{lll}
\hline
 & Untreated & Metoprolol \\
 & \((n=10)\) & \((n=10)\) & \(p^*\) \\
\hline
Permeability \((\mu l/hr/cm^2)\) & \\
Coronary artery & 0.199±0.33 & 0.073±0.05 & NS \\
R carotid artery & 0.037±0.02 & 0.060±0.05 & <0.09 \\
R carotid bifurcation & 0.096±0.06 & 0.124±0.08 & NS \\
Cerebral arteries & 0.006±0.007 & 0.005±0.005 & NS \\
Abdominal aorta & 0.150±0.06 & 0.176±0.08 & NS \\
Concentration \((\mu g/cholesterol/g)\) & \\
Coronary arteries & 33.6±10.9 & 36.4±28.7 & NS \\
R carotid artery & 21.05±16.7 & 32.45±23.5 & NS \\
R carotid bifurcation & 54.9±43.8 & 54.8±34.3 & NS \\
Cerebral arteries & 2.8±4.2 & 5.1±9.7 & NS \\
Abdominal aorta & 27.7±17.1 & 11.8±5 & <0.02 \\
\hline
\end{tabular}
\end{table}

Values are mean±SD. NS, not significant; R, right.

*One-factor analysis of covariance was used to evaluate the effects of treatment (untreated vs. metoprolol-treated groups), with heart rate in the pre-experimental period as a covariate.
this same site. We did not assess the arterial permeability to LDL in the thoracic aorta, another arterial site that is susceptible to aggravation of atherosclerosis by psychosocial stress and in which we found evidence of endothelial injury.

In summary, this study demonstrated that monkeys subjected to acute psychological stress show evidence of endothelial cell injury that can be suppressed by simultaneous treatment with the β-blocker metprolol. It is possible that the absence of differences in leukocyte adhesion or interaction of LDL with artery can be attributed to biological variation among animals, preventing detection of small differences. Alternatively, however, it may be that exacerbation of atherosclerosis by psychological stress is mediated, at least initially, by endothelial injury. If so, alterations in leukocyte adhesion or interaction of LDL with artery might not have occurred, given the relatively acute nature of the manipulation to which the monkeys were exposed, and the absence of diet high in saturated fat and cholesterol. Future studies will be required to resolve these issues.

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KEY WORDS • atherosclerosis • heart rate • sympathetic nervous system
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