Lipoproteins and the Inhibitory Effect of Human Endothelial Cells on Platelet Function

ARNE NORDØY, BIRGIT SVENSSON, DONALD WIEBE, AND JOHN C. HOAK

SUMMARY We investigated the effect of plasma low density lipoproteins (LDL), very low density lipoproteins (VLDL), and high density lipoproteins (HDL) on the platelet inhibitory effect of primary cultures of human endothelial cell monolayers (ECM). ECM incubated with lipoprotein-deficient plasma (LDP) for 2 hours at 37°C had an inhibitory effect on ADP- and collagen-induced platelet aggregation and prostaglandin production in platelet-rich plasma similar to that observed when ECM were preincubated with growth medium or plasma. Concentrations of LDL in LDP up to a protein concentration of 1600 µg/ml had an inhibitory effect on the endothelial cells' ability to modulate these platelet reactions. VLDL at the highest concentration (1600 µg/ml) had a slightly inhibitory effect, whereas HDL showed no such effect. The inhibitory effect of LDL was not observed during the first hour of incubation. When HDL in concentrations similar to or higher than LDL were combined with LDL, the inhibitory effect of LDL was partially reduced. VLDL combined with LDL or HDL did not interfere with the effects of the later fractions. The inhibitory effect of LDL was significantly reduced when LDL were diluted in whole plasma. Prostacyclin which is synthesized and released from the endothelial cells and contributes to the inhibitory effect upon platelets did not change its effect on platelet reactivity by preincubation with the various lipoprotein fractions. The current studies may indicate that LDL have a direct effect on the endothelial cells and that this effect may be partially counteracted by HDL. This effect of LDL on the endothelial cells reduces the endothelium's ability to inhibit platelet aggregation and thus could favor the tendency to thrombus formation.

Recent studies have shown that microsomal fractions of the vessel wall lining of arteries and veins are able to generate prostacyclin (PGI₂) from prostaglandin endoperoxides and arachidonic acid. PGI₂ has a strong inhibitory effect on platelet aggregation induced by collagen, ADP, and epinephrine. It has been shown further that human endothelial cells grow in culture spontaneously release a platelet antiaggregatory principle that also inhibits malondialdehyde (MDA) production in platelets induced by collagen. These observations suggest that a normally functioning endothelial cell is able to counteract platelet aggregation and thrombus formation, and that this effect is caused partially by PGI₂ through an inhibition of thromboxane A₂ (TXA₂) generation in platelets, which is the strongest naturally occurring platelet-aggregating compound described.

Epidemiological studies have shown a positive correlation between the plasma concentration of low density lipoproteins (LDL) and the likelihood of a person developing clinical coronary heart disease. In some reports, a similar correlation has been established for the plasma concentration of very low density lipoproteins (VLDL). Plasma high density lipoprotein (HDL) concentrations have been independently, inversely related to coronary risk. Thrombosis is a common finding in patients dying of coronary heart disease; however, the mechanisms relating changes in plasma lipoproteins to an increased thrombosis tendency have been only poorly defined.

Patients with coronary heart disease and subjects with asymptomatic familial hyperbetalipoproteinemia have been reported to have increased platelet consumption. Subjects with type II hyperlipoproteinemia have demonstrated increased sensitivity to platelet-aggregating substances, which seems to be associated with increased TXA₂ formation in the platelets. Ross and Harker have demonstrated a focal loss of endothelial cells in monkeys with a diet-induced hypercholesterolemia, and cross-over platelet survival studies showed a shortened survival of platelets obtained from both normal and hypercholesterolemic animals when infused into hyperlipemic monkeys.

Studies using cultures of human and rabbit endothelial cells have shown binding, uptake, and degradation of LDL by the cells. HDL reduced the uptake of LDL and affected binding to a lesser extent. Bovine aortic endothelial cells have also
demonstrated uptake of VLDL. Exposure of endothelial cells to LDL suspended in a culture medium caused extensive morphological changes which were prevented when lipoprotein-poor serum was added.

The present study reports on the effect of the main lipoprotein fractions, alone and in combination, on the platelet-inhibitory effect of primary cultures of human endothelial cells.

Methods

Materials

Medium 199, Hanks' balanced salt solution, vitamin solution, amino acids solution, and L-glutamine were purchased from Grand Island Biological Co. Trizma base (Tris), HEPES buffer, tendon collagen, and disodium adenosine diphosphate (ADP) were obtained from Sigma Chemical Co. Prostacyclin (PGI₂) was kindly given to us by Dr. K.C. Nicolaou, Department of Chemistry, University of Pennsylvania. Culture flasks, Petri dishes, and platelet centrifuge tubes were purchased from Falcon Plastics. Malondialdehyde tetramethyl acetal was purchased from Eastman Kodak. Modified medium 199 (NM-100) contained: medium 199; 2XBBME vitamin solution; 2XBBME amino acids solution, glucose, 2 g/liter; neomycin, 0.1 g/liter; and 20% fetal calf serum, pH 7.4. Modified Hanks' balanced salt solution (MHBSS) was prepared by dissolving in distilled water 3.574 g of HEPES buffer (15 mM final concentration), 100 ml (10x) of Hanks' balanced salt solution without NaHCO₃, and 10 ml (100x) of L-glutamine (200 mM). The pH was adjusted to 7.4 with 1 N NaOH and, after dilution to 1000 ml final volume with distilled water, the medium was sterilized by filtration through a 0.22-μm sterile Millipore filter. Tris-buffered saline (TBS) contained 139 mM NaCl and 15 mM Tris pH 7.4. ADP: a stock solution of 3 × 10⁻³ M in TBS was stored at −20°C and was used within 4 hours. Collagen suspension was prepared in diluted acetic acid as earlier described. PGI₂ (1 mM) was dissolved in 0.1 M sodium carbonate and kept at −20°C until use, when it was further diluted in 0.1 M sodium carbonate and 0.05 M Tris buffer (TB), pH 7.4.

Isolation and Culture of Cells

Primary cultures of endothelial cells were prepared from human umbilical veins according to a slight modification of the method of Jaffe et al. Confluent primary endothelial cell monolayers (ECM) were used for further studies 4 days after the Petri dishes were seeded. Empty dishes (ED) washed with the culture medium were used as controls. Eighteen to 24 hours prior to the addition of lipoproteins, the cell layers were washed two times with TBS and the cells were fed the culture medium in which lipoprotein-deficient human serum (LDS) at a protein concentration of 40 mg/ml replaced fetal calf serum.

Lipoproteins

Human LDL [density (d.) 1.019–1.063 g/ml], VLDL (d. <1.006 g/ml), HDL (d. 1.063–1.215 g/ml) and lipoprotein-deficient plasma (LDP) (d. >1.215 g/ml) were prepared from single 500-ml units of blood collected in 63 ml of CPD (206 mg of citric acid, 1.66 g of sodium citrate, 140 mg of sodium biphosphate, and 1.61 g of dextrose in 1000 ml of solution) from normolipemic healthy donors. Lipoproteins were fractionated by sequential flotation in a Beckman preparative ultracentrifuge at 100,000 g (average) for 18–20 hours at 15°C according to standard techniques. Initially, plasma was spun at a density of 1,006 g/ml for VLDL isolation. After each spin, the first 5- to 7-ml fraction was collected and the next 5- to 10-ml sample was removed to avoid contamination. The LDL, HDL, and LDP fractions were dialyzed against 0.15 M NaCl for 48 hours at 4°C with a minimum of three changes of 200 volumes of 0.15 M NaCl. All fractions were analyzed for cholesterol, triglyceride, and protein. Prior to the protein determination of the HDL fraction, a 1-ml sample was spun at 1,210 g/ml density at an average of 100,000 g at 15°C for 20 hours to remove extraneous proteins. Each isolated lipoprotein fraction migrated as a homogeneous peak on lipoprotein electrophoresis. HDL cholesterol in plasma was measured as recently described. LDS was prepared from LDP by adding human thrombin (10 U/ml), stirring with a glass rod for 10 minutes at 37°C, removing fibrin, and centrifuging for 1 hour at 30,000 g. The supernatant fluid was sterilized by filtration through a 0.22-μm Millipore filter. The lipoprotein fractions maintained their biological activity as measured by their effect on the the ECM during the 4-week storage period, and the lipoprotein fractions similarly maintained their appearance as a homogeneous peak on electrophoresis at the end of the 4 weeks.

Platelet Studies

Venous blood was collected from healthy, fasting, normolipemic donors who had not taken any drugs for the last 14 days. Nine volumes of blood were collected in one volume of 3.2% trisodium citrate dihydrate, and platelet-rich (PRP) and platelet-poor (PPP) plasma were prepared by centrifugation at 20°C at 270 g for 15 minutes and 1200 g for 30 minutes, respectively. The platelet number in PRP was adjusted to 300,000/μl by the addition of PPP. PRP was kept at 22°C and was used within 4 hours after sampling.
Platelet aggregation was recorded according to the method of Born using a Payton aggregometer module (Payton Assoc.) at 37°C. The percentage of aggregation registered after 1 minute of preincubation and an additional 6 minutes after addition of the aggregation agent was used as the endpoint of aggregation, using the light transmission (OD) of PPP as 100% and that of PRP as 0%. Final concentrations of 10 μM ADP and collagen, 70 μg/ml, were used throughout. Platelets were counted in a Coulter Counter, (Coulter Electronics). MDA was measured in PRP samples from the aggregometer 6 minutes after the addition of collagen, according to the method of Smith et al. The significance of differences between means was based on Student's t-test for paired samples.

**Test Procedure**

The growth medium was aspirated from the ECM in 35- × 10-mm Petri dishes and replaced with the various lipoprotein fractions diluted in LDP or PPP to the desired concentration. The ECM was then incubated for the desired length of time, usually 2 hours at 37°C in a metabolic incubator. The dishes were placed on a rocker platform with a 30° tilt (Bellco Glass, Inc.) and rocked continuously at a rate of 10 up-and-down cycles per minute for the initial 15 minutes of the incubation period. At the end of the incubation period, the lipoprotein solution was pipetted off and the dishes were washed three times in 1 ml of MHBSS. After the washing procedure, the dishes were always inspected with a microscope to ensure the persistence of confluent monolayers. Occasionally (<3%), detachment of the endothelial cells from the dishes was observed and these cultures were discarded. The detachment was not related to incubation with any specific lipoprotein fraction or to the concentration of the actual lipoprotein fraction. The intact monolayers were then incubated with 1.5 ml of TB for another 5 minutes on the rocker at 37°C. A volume of 0.3 ml of the TB was aspirated from the dishes and added to 0.5 ml PRP, and ADP-induced platelet aggregation (PA) in this mixture was recorded. The rest of the TB was aspirated from the dishes and discarded, and the ECM was then incubated with 1.5 ml of PRP for 30 minutes on the rocker at 37°C. A volume of 0.9 ml of this PRP was collected, and collagen-induced PA and MDA production were measured.

**Statistics**

The significance of differences between means were based on Student's t-test for paired samples.

**Results**

The endothelial cells incubated with LDS in the medium for the last 24 hours showed an inhibitory effect on ADP-induced and collagen-induced platelet aggregation and MDA production in PRP similar to that observed when the traditional medium with calf serum was used. The Effect of Lipoproteins Diluted in LDP.

When the endothelial cells were incubated for 2 hours with LDL diluted in LDP to give final concentrations of protein of 200–1600 μg/ml, a gradual decrease in the platelet-inhibitory effect of the cells was observed (Table 1). The reactivity against collagen-induced PA and MDA production was not significantly different from that observed when TB or PRP was incubated with Petri dishes without endothelial cells (ED). Occasionally, the ECM seemed to be resistant to incubation of LDL even at high concentrations. The inhibitory effect of LDL (1600 μg/ml) was subsequently tested using 13 different cultures and PRP collected from a similar number of donors. The control cultures preincubated with LDL showed an inhibition of ADP-induced PA of 82 ± 4%, whereas those preincubated with LDL showed 44 ± 8% (P < 0.01). However, 4 of the 13 cultures showed less than 10% difference of the inhibitory effect between those preincubated with or without LDL (Fig. 1). The inhibitory effect of endothelial cells on collagen-induced PA and MDA production was more consistently affected by preincubation with LDL. However, 3 of the 13 cultures showed, even without preincubation with LDL, a very low, if any, inhibitory effect on platelet reactivity with regard to their response to collagen (Fig. 1).

When ECM was preincubated with HDL in concentrations similar to or higher than LDL, no significant loss of the inhibitory effect of the endothelial cells was observed (Table 1). VLDL diluted in LDP showed no significant inhibitory effect on the endothelial cells with regard to their ability to inhibit ADP-induced PA. However, at the highest concentration (1600 μg/ml protein), VLDL had a moderate inhibitory effect on the endothelial cells' ability to counteract the effect of collagen on platelets (Table 1).

To explore further the effect of LDL on the endothelial cells, the ECM were incubated with LDL (1600 μg/ml) for varying periods of times. As shown in Table 2, no significant inhibition was observed during the first hour of incubation.

The Effect of Combined Lipoprotein Fractions Diluted in LDP.

When ECM was incubated with HDL in addition to LDL in concentrations that alone would affect the behavior of the endothelial cells, a partial reduction of the inhibitory effect of LDL was observed (Table 3). The combination of VLDL and LDL did not significantly interfere with the reactivity of the endothelial cells compared with the effect of LDL alone (Table 4). Similarly, no significant changes were observed when VLDL and HDL were com-
Table 1  The Effect of Low Density Lipoproteins (LDL), High Density Lipoproteins (HDL), and Very Low Density Lipoproteins (VLDL) on Endothelial Cell Monolayers (ECM)

<table>
<thead>
<tr>
<th>Lipoproteins (µg/ml)</th>
<th>FA (%) (ADP 10 µM)</th>
<th>FA (%) (collagen 70 µg/ml)</th>
<th>MDA (ng/ml)</th>
</tr>
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<tbody>
<tr>
<td>A. LDL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>50 ± 8*</td>
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<td>50 ± 8</td>
<td>46.1 ± 8.3</td>
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<td>18 ± 5†</td>
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<td>84 ± 2*</td>
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<tr>
<td>B. HDL</td>
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<td></td>
<td></td>
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<tr>
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<td>50 ± 8*</td>
<td>79 ± 3*</td>
<td>66.8 ± 8.7*</td>
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<td>60 ± 10</td>
<td>62.2 ± 11.8</td>
</tr>
<tr>
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<td>3 ± 1†</td>
<td>69 ± 5†</td>
<td>60.0 ± 12.4‡</td>
</tr>
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<td>62 ± 9†</td>
<td>64.2 ± 9.5‡</td>
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<td>1600</td>
<td>20 ± 12‡</td>
<td>77 ± 2†</td>
<td>73.5 ± 10.5§</td>
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All results are expressed as mean ± SEM. A = results of 7 experiments; B = results of 7 experiments (except A = results of 4 experiments); C = results of 6 experiments.

Endothelial Cells Preincubated with LDL, HDL, and VLDL diluted in lipoprotein-deficient plasma (LDP) at 37°C for 2 hours. The ECM were washed 3 times, and the inhibitory effects of ECM on ADP- and collagen-induced platelet aggregation (PA) and malondialdehyde (MDA) production in citrated platelet-rich plasma were recorded. Controls in empty dish (ED). Significance of difference between the mean and the mean recorded when ECM were preincubated with LDP (0) is as follows:

* P < 0.001; † P < 0.05; ‡ not significant; § P < 0.01; ¶ P < 0.10, > 0.05.

Figure 1  The inhibitory effect of endothelial cell monolayers incubated for 2 hours at 37°C with lipoprotein-deficient plasma or low density lipoproteins at a protein concentration of 1600 µg/ml, on ADP- (10 µM) and collagen (70 µg/ml) induced platelet aggregation and collagen-induced malondialdehyde (MDA) production in platelet-rich plasma.
of preincubation at 37°C, ADP-induced PA was tested. Control samples were aliquots of lipoprotein fractions added to the solvent without PGI₂. No significant difference of the inhibitory effect of PGI₂ dissolved in the different lipoprotein fractions was noted (Table 6).

**Discussion**

The present study has confirmed that primary cultures of endothelial cells prepared from human umbilical veins have a spontaneous inhibitory effect on ADP- and collagen-induced platelet aggregation and MDA production in citrated platelet-rich plasma. This inhibitory effect was reduced by incubation of the endothelial cells with LDL prepared from normolipemic subjects and was observed after a 2-hour incubation period. Occasionally, the endothelial cells maintained their inhibitory effect after incubation with LDL. This lack of response was not related to the source of lipoproteins because it was observed with lipoprotein fractions prepared from three different donors with normal plasma cholesterol and triglyceride concentrations. It could be related to the reactivity of the individual cell cultures to LDL, since their spontaneous inhibitory effect on platelets did not differ from that of the other cultures.

Previous studies have shown that endothelial cells bind LDL to high- and low-affinity binding sites, followed by internalization and degradation with release of acid-soluble material. The present observations indicate that the inhibitory effects of LDL on the function of the endothelial cells were not related to binding of LDL to the high-affinity sites which occurs after a short incubation period and at very low concentrations. Furthermore, it seems unlikely that the effects were caused by release of degradation products because all cultures were washed three times after the incubation period and before the effect on platelet reactivity was tested. HDL had no inhibitory effect on the function of the endothelial cells; in contrast they were able, at least partially, to counteract the effect of LDL. The observation that LDL diluted in whole plasma in many cases also was without effect on the endothelial cells may indicate that the plasma HDL, similar to the purified HDL fractions, modified the interaction between LDL and endothelial cells. The plasma samples would contribute about 900 μg/ml HDL protein to the incubation mixture. Stein and Stein used LDL concentrations similar to those in this study and showed that the total uptake of LDL and its degradation products was markedly reduced, whereas the binding was affected only slightly by HDL. In studies with human fibroblasts, a 3-fold increase of surface binding of LDL occurred when the cells were maintained on a lipoprotein-deficient medium. Very low density lipoproteins had only a very moderate inhibitory effect on the endothelial cells and did not seem to potentiate the effect of LDL.

It is not clear how high concentrations of LDL could directly inhibit the mechanisms in the endothelial cells and did not seem to potentiate the effect of LDL.
adenylate cyclase in plasma membranes of many cells, indicating that still undefined mechanisms may be involved.38

Proteins have been shown to activate enzymes like regard to production site and nature.34-37 Lipoproteins suggested for prostaglandin synthesis in human platelets.17 Furthermore, substances other than the platelet inhibitors of platelet aggregation may indicate that LDL inhibits synthesis or release of these compounds. It has been shown that microsomal fractions prepared from intima-media layers of aorta from rabbits that were fed an atherogenic diet, and that had actually developed atherosclerosis, showed increased transformation of arachidonic acid to PGE2, a prostaglandin that aggregates the endothelial cells grown in culture, even after 2 hours of incubation with a concentration of LDL that would be considered high, but not excessively so, in humans, show functional disturbances that did not occur on incubation with the other lipoprotein fractions at similar concentrations. Such changes in the function of the endothelial cells with reduced ability to inhibit platelet aggregation and prostaglandin synthesis in platelets could eventually favor thrombus formation. Prospective9,43 and retrospective44,45 studies on humans have confirmed that susceptibility to coronary heart disease is positively correlated with the plasma LDL concentration and negatively correlated to the plasma concentration of HDL. It can be suggested that the very early changes in the vessel wall in patients with hyperlipoproteinemia may be related to lipoprotein-endothelial interactions with subsequent inhibition of the normal function of the vessel wall.

Studies on animals have shown that, after a short period on a hypercholesterolemic diet (less than 14 days) which regularly is accompanied by a marked increase of LDL concentration in plasma, many species show significant changes in the endothelial cells.39-42 These changes were associated with shortened platelet survival which returned to normal at the time of reendothelialization.

The present studies indicate that human endothelial cells grown in culture, even after 2 hours of incubation with a concentration of LDL that would be considered high, but not excessively so, in humans, show functional disturbances that did not occur on incubation with the other lipoprotein fractions at similar concentrations. Such changes in the function of the endothelial cells with reduced ability to inhibit platelet aggregation and prostaglandin synthesis in platelets could eventually favor thrombus formation. Prospective9,43 and retrospective44,45 studies on humans have confirmed that susceptibility to coronary heart disease is positively correlated with the plasma LDL concentration and negatively correlated to the plasma concentration of HDL. It can be suggested that the very early changes in the vessel wall in patients with hyperlipoproteinemia may be related to lipoprotein-endothelial interactions with subsequent inhibition of the normal function of the vessel wall.

### Table 4 The Combined Effect of VLDL and HDL and of VLDL and LDL on Endothelial Cell Monolayers

<table>
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<tr>
<th>VLDL (µg/ml)</th>
<th>HDL (µg/ml)</th>
<th>LDL (µg/ml)</th>
<th>No.</th>
<th>PA (%) (ADP 10 µM)</th>
<th>P*</th>
<th>PA (%) (collagen 70 µg/ml)</th>
<th>P*</th>
<th>MDA (ng/ml)</th>
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<td>0 (ED)</td>
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*Results in columns 5, 7, and 9 are expressed as mean ± SEM. Procedure was as given in Table 1. NS = not significant.

†A second series of 6 experiments.

### Table 5 The Effect of LDL Diluted in Platelet-Poor Plasma on the Inhibitory Effect of Endothelial Cell Monolayers

<table>
<thead>
<tr>
<th>LDL (µg/ml)</th>
<th>PA (%) (ADP 10 µM)</th>
<th>P*</th>
<th>PA (%) (collagen 70 µg/ml)</th>
<th>P*</th>
<th>MDA (ng/ml)</th>
<th>P*</th>
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</tr>
<tr>
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</tr>
</tbody>
</table>

*Results in columns 2, 4, and 6 are mean ± SEM of 10 experiments. Procedure was as given in Table 1. NS = not significant.

†As in Table 1.
References


38. Paireault J, Levilliers J, Chapman MJ: Human serum lipo-
SUMMARY  There are afferent nerve fibers responsive to alterations of the kidney's chemical environment in the renal nerves of the rat. In anesthetized, artificially ventilated, male Sprague-Dawley rats, single unit recordings were prepared by dissection of the centrally cut nerves of the right kidney. The stimuli used included occlusion of the renal artery, systemic asphyxia, changes in renal arterial and venous pressures, changes in ureteral pressure, and cyanide infusion. We found a population of sensory nerve fibers whose endings are activated only during markedly impaired renal blood flow (produced by clamping the renal artery, severe hypotension below 40 mm Hg, and prolonged occlusion of the renal vein), and during systemic asphyxia. The same units are not responsive to increases and decreases in systemic arterial pressure (range: 40–190 mm Hg), to ureteral pressure (range: 0–50 mm Hg), or to changes in renal venous pressure. None of the 40 single units studied was spontaneously active; their pattern of activation during renal ischemia always was characterized by trains of impulses. These sensory units have functional properties distinctly different from those of known renal mechanoreceptors. They appear to be a homogeneous group of sensory elements, and we have termed them renal ("R") chemoreceptors. Evidence also is presented which is consistent with the concept that a chemical substance released by or accumulated within the kidney might be the agent activating these chemoreceptors during renal ischemia.

ACTION potentials initiated by the activation of sensory nerve fibers in the kidney have been recorded from the renal nerves of rats,1 cats,2–4 dogs,5 and rabbits.6–8 The renal receptors thus disclosed respond only to changes in intrarenal pressure and have been termed renal mechanoreceptors. In two studies, occlusion of the renal artery was observed to activate a group of fibers different from those sensitive to intrarenal pressure;3 however, no attempts were made to characterize this group further.

The present report describes a population of afferent fibers from the kidney responsive to renal ischemia but not to changes in intrarenal pressure. We found suggestive evidence that the stimulus responsible for their activation is the release or accumulation of a chemical substance inside the ischemic kidney. To underline their peculiar functional properties and to differentiate them from the well-known carotid and aortic body chemoreceptors, we have termed the renal nerve endings sensitive to ischemia renal ("R") chemoreceptors.

**Methods**

We studied 50 male Sprague-Dawley rats (150–250 g) bred in our laboratories. They were anesthetized by intraperitoneal injection of sodium pentobarbital, 5 mg/100 g (40 rats), or Inactin, 12.5 mg/100 g (10 rats). After a paralyzing dose of gallamine triethiodide, 2 mg/100 g, iv, the rats were maintained by intraperitoneal pentobarbital, 5 mg/100 g (40 rats), or Inactin, 12.5 mg/100 g (10 rats). After a paralyzing dose of gallamine triethiodide, 2 mg/100 g, iv, the rats were ventilated artificially. The respirator was adjusted to maintain arterial Po2, Pco2, and pH within normal limits as tested with a Radiometer blood gas analyzer (BMS 3 MK2). The rats were maintained at 37°C on a heated operating table.

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