Enhanced Accumulation and Turnover of Esterified Cholesterol in Injured Rabbit Aorta

Dawn C. Schwenke and Donald B. Zilversmit

Arterial injury exacerbatess experimental atherogenesis. This report evaluates cholesteryl ester influx and loss during the first 5 days after arterial injury. Selected areas of aortas from rabbits made hypercholesterolemic by 12 to 16 days of cholesterol feeding were injured with a balloon catheter. This allowed measurements and a relatively precise comparison of cholesterol and cholesteryl ester influx into the uninjured and injured arterial wall within the same animal. The animals received oral doses of \(^{3}H\)- or \(^{14}C\)-cholesterol either just before injury or 1 day later. Most animals were given the other isotope of cholesterol 1 day before sacrifice, which took place 2 to 5 days after injury. Measurement of accumulated labelled esterified cholesterol within the same animal during two different time periods allowed the estimation of total influx and fractional loss of enterestefied cholesterol within the artery. Between 2 and 5 days after injury, total influx into areas of injured artery averaged 30 to 60 times the total influx into the adjacent uninjured areas. By the fifth day after injury, the esterified cholesterol content was about 10 times greater in injured areas than in adjacent, uninjured areas. The nonesterified cholesterol content of injured areas was about 70% greater than that of adjacent noninjured areas. Total influx and fractional loss of arterial cholesteryl ester calculated from radioactivity data could account for \(117 \pm 6\) (mean \(\pm\) SEM) percent of the cholesteryl ester mass that accumulated during the interval from injury to sacrifice. The total influx rates and daily increments in cholesteryl ester content of injured areas were positively related.

(Arteriosclerosis 7:367–377, July/August 1987)

The enhancement of atherosclerosis by arterial injury in either the presence\(^1,2,3\) or absence\(^1,2,3,4\) of cholesterol and/or fat feeding has been well documented. This effect is not limited to a single type of injury; it is observed in cases of immune injury,\(^5\) chemical injury,\(^6\) and mechanical injury.\(^1,2,3,4,7\) Most importantly, lesions resulting from arterial injury in animals resemble human lesions more closely than those produced in animals by cholesterol feeding alone.\(^1,4,5\)

Several hypotheses have been advanced as possible explanations for the effects of injury. One of these\(^8\) is that removal of the endothelium eliminates a barrier to influx of macromolecules from plasma. Others\(^8\) argue that the endothelium prevents efflux of macromolecules from the artery. At present, relatively few studies have considered the contribution of plasma cholesteryl ester influx to the observed excess cholesteryl ester accumulating in the artery after injury. Most of the measurements of cholesteryl ester influx\(^3\) or lipoprotein accumulation\(^10,11\) into previously injured areas of the arterial wall have been made at relatively long times (months) after injury. At these times, the composition of the arterial wall in both reendothelialized areas and those remaining deendothelialized is considerably altered compared with uninjured areas.\(^2,7,12,13\) Several studies suggest that the atherosclerosis risk factors, hypercholesterolemia\(^14,15\) and hypertension,\(^16\) injure the arterial endothelium.

Other studies\(^10\) have measured the ability of acutely deendothelialized arterial tissue to limit the accumulation of low density lipoprotein (LDL) in vivo. Yet others\(^17\) have studied the uptake of LDL by perfused vessels. The present investigation addresses the question of whether removal of aortic endothelium and perhaps underlying elements affect cholesteryl ester accumulation primarily by reducing the barrier to influx or to efflux of cholesteryl ester, or both. Specifically we have asked: 1) Does injury to the luminal surface of the artery result in enhanced cholesteryl ester influx in the injured artery? 2) If cholesteryl ester influx is elevated in injured areas, does the cholesteryl ester content of these areas increase concomitantly with the enhanced influx? 3) Is cholesteryl ester turnover altered in injured areas?

Methods

Chemicals

All chemicals were reagent grade. Cholesterol methyl ether, 99% pure, (\(\Delta^5\)-cholest-3-en-30-ol-30-methyl) was pur-
chased from Sigma Chemical Company (St. Louis, Missouri). Also purchased from Sigma were rabbit serum albumin (crystalized and lyophilized), cholesteryl oleate, and heparin (grade 1, sodium salt). Cholesterol used as an assay standard was purchased from ICN Biochemical (Cleveland, Ohio) and was purified by crystallization as the dibromide. Evans blue dye (25 mg/5 ml ampul in distilled water) was obtained from Harvey Laboratories, Incorporated (Philadelphia, Pennsylvania). Several anesthetics were used: sodium pentobarbital (Premo Pharmaceutical Laboratories, Incorporated, Hackensack, New Jersey), ketamine hydrochloride (Ketaset, Bristol Laboratories, Syracuse, New York) and xylazine (Rompun, Haver-Lockhart, Pennsauken, New Jersey). Solvents were freshly distilled and glassware was washed with acid before use.

Isotopes

Labelled materials, $^{1}a_{2},2a(n)-^{3}H$-cholesterol, 60 Ci/mmol; $^{4}^{14}C$-cholesterol, 58.4 mCi/mmol; $^{125}$I as NaI in dilute NaOH solution (pH 7 to 11), 1.8 Ci/μmol NaI; and Na$_{51}$CrO$_{4}$ in 0.15 M NaCl (pH 5 to 8), 12.5 to 25.0 mCi/μmol Cr were obtained from Amersham Corporation (Arlington Heights, Illinois). In most cases, the labelled cholesterol was more than 95% pure by thin-layer chromatography (TLC) on precoated silica gel plates (Silica Gel 60, E. Merck, Cherry Hill, New Jersey) developed with 50:50:1 (vol/vol/vol) hexane/diethyl ether/acetic acid.

Animals

Female New Zealand white rabbits, obtained from Becken's Research Animal Farm (Sanborn, New York), were used in all experiments. The rabbits were initially fed 100 g of Rabbit Laboratory Chow (Ralston Purina, St. Louis, Missouri). Beginning 12 to 16 days before endothelial injury, the diet was supplemented with 0.5% or 1% cholesterol and 2.5% Wesson Oil (Hunt Wesson Foods, Inc., Fullerton, California). Cholesterol feeding was continued until sacrifice 1 to 5 days after injury. Experimental protocols, including the dosage of anesthetics used for surgery and euthanasia, conformed to Cornell University guidelines.

Injury

The aortas of rabbits weighing 2.72 ± 0.04 kg (mean ± SEM, n = 24) were injured by a modification of the method of Baumgartner under aseptic conditions. Anesthesia was induced with 25 mg sodium pentobarbital intravenously and supplemental diethyl ether, or 35 mg ketamine hydrochloride and 5 mg xylazine intramuscularly/kg body weight. A 4F thin-walled embolectomy catheter (Edwards Laboratory, Division of American Hospital Supply, Santa Ana, California) was introduced via a femoral arteriotomy into the thoracic aorta to about the level of the second pair of intercostal arteries. The balloon was inflated with saline to 700 mm Hg with an infusion pump (Multi-Speed Transmission, Model #600-000, Harvard Apparatus Company, Dover, Massachusetts). The inflated balloon was drawn caudally about 4 cm while turning 1 to 2 revolutions and then was deflated. The catheter was readvanced to the cephalic edge of the injury and the process was usually repeated to abrade the area a total of three times. The catheter and deflated balloon were then withdrawn about 4 cm caudally before injuring a second site, beginning 0 to 2 cm proximal to the renal arteries (see Figure 1). In general, the central uninjured area contained the orifice to the celiac artery as well as one to two other orifices. The time required to inflate the balloon was 20 to 45 seconds; the total time needed to complete the injuries was about 12 minutes. The femoral artery was ligated and the wound was sutured. All of the animals tolerated the procedure well; neither sepsis nor mortality occurred.

Characterization of the Injury

Three days after injury, the aortas of three animals were fixed in situ for histological assessment of the extent of damage caused by the ballooning procedure. One ampul of Evans blue dye was given intravenously 1 to 3 hours before sacrifice in order to localize injured and noninjured areas. Under ketamine hydrochloride and xylazine anesthesia, 35 and 5 mg/kg, respectively, 1000 to 2000 IU heparin was injected intravenously. The left carotid artery was cannulated and the femoral veins were severed.

Figure 1. The location of the injured areas of the aorta as visualized by Evans blue dye. Hatched regions denote injured areas stained by the dye. The dotted line indicates the point at which (in some cases) the arch was separated from the proximal thoracic aorta. The lines in the central and distal control regions indicate focal areas of Evans blue dye uptake, presumably due to scratching of these areas by the uninflated balloon.
Blood was flushed from the arterial tree by perfusion via the carotid cannula with 0.1 M sodium phosphate buffer (pH 7.4) at a pressure of 90 to 110 mm Hg for about 10 minutes. Next, fixation at the same pressure was started with 10% formaldehyde in the same buffer. After fixation in situ for 30 minutes, the aorta was removed and fixation was continued by immersion for a further 24 hours. Samples from injured and uninjured areas were dehydrated by transferring through a graded series of alcohol into toluene. Paraffin sections were stained with hematoxylin and eosin or Verhoeff's elastic stain and were then counterstained by the Van Gieson technique.

**Preparation of the Radioactive Doses**

Oral doses were prepared by adsorbing either 3H-cholesterol (150 to 500 μCi) or 14C-cholesterol (15 to 50 μCi), along with 1 mg cholesterol carrier, from diethyl ether onto 25 to 50 g of the cholesterol-enriched diet. The labelled diets were dried in a hood overnight before use.

**Calculation of Total Influx and Fractional Loss of Entered Cholesteryl Ester**

We have considered cholesteryl ester in the injured artery as the sum of an inert pool of cholesteryl ester present before injury and a second, well-mixed, labile pool of cholesteryl ester entering after injury. Because the arterial cholesteryl ester mass increased after injury, we considered the injured artery to be in a nonsteady state. We assumed that the labile arterial cholesteryl ester could be approximated by an open, one-compartmental model. The fraction of the plasma cholesteryl ester pool entering the artery per unit time, and the fraction of the labile cholesteryl ester pool lost from the injured artery per unit time, were considered approximately constant during the experimental period.

By measuring the arterial accumulation of two differently labelled forms of cholesteryl ester after two different time intervals within the same animal, we were able to calculate the total influx (influx corrected for loss of entered cholesteryl ester) and the fractional loss of cholesteryl ester. The total influx (or total cholesteryl ester influx) and the fractional loss of cholesteryl ester were calculated from equations derived in the appendix and from the coefficients of the polynomials fitted to the plasma cholesteryl ester radioactivity curves (Figure 2). We also calculated the influx of cholesteryl ester as arterial cholesteryl ester radioactivity divided by the area under the plasma cholesteryl ester radioactivity curve (sink assumption). This quantity is designated as retained cholesteryl ester influx, or retained influx.

**Protocol for Influx Experiments**

In most cases, the animals were fed two differently labelled forms of cholesterol. Two animals were fed the first dose of labelled cholesterol 1 to 2 hours before injury and the second dose 1 day later. Eleven other animals were fed the first dose of labelled cholesterol 1 day after injury. Seven of these animals received the second meal of labelled cholesterol diet 1 day later. The last four rabbits were fed the second cholesterol label 3 days after being fed the first label. Each animal was sacrificed 1 day after being fed the second cholesterol label. Thus, in the aortas studied 2, 3, and 5 days after injury, the arterial labelled cholesterol accumulated during the last 1 and 2, 1 and 2, and 1 and 4 days of life, respectively. The rabbits were fed diets labelled with either 3H- or 14C-cholesterol as described above. After consuming the labelled diet, each rabbit received the remainder of its daily 100 g ration as unlabelled cholesterol-enriched diet. Serial blood samples were collected into 0.01 volume of 0.4 M EDTA, 4% azide in 0.15 M NaCl (pH 7.4) until the rabbits were sacrificed. Half of the rabbits received 3H- and then 14C-cholesterol; the other half received the doses in the reverse order. Two other rabbits sacrificed 2 days after injury received only one dose of labelled cholesterol. Six more rabbits that were sacrificed 1 day after injury received only labelled albumin or labelled red cells (below); these animals provided cholesterol mass data, but no calculated values of total influx and fractional loss.

**Sacrifice and Tissue Isolation**

At 1 to 3 hours after injection of Evans blue dye, the animals were deeply anesthetized with sodium pentobarbital, their chests were opened, and their arterial systems were perfused via the left ventricle with 500 to 600 ml 0.15 M NaCl at 80 mm Hg. The aorta, from aortic valve to iliac bifurcation, was removed, rinsed with 0.15 M NaCl, and kept on ice until removal of adventitial debris. After the aorta was opened, the blue-white boundaries were marked on paper and in most cases the aorta was photographed. The surface areas of the injured and noninjured regions were measured by planimetry of the paper tracings.

The blue (injured) and white (noninjured) areas were...
separated, as illustrated in Figure 1. The inner layer of artery, containing the intima and some media, was stripped (Bergh cilia forceps, cat #MX18-1108, Miltex, Lake Success, New York; kindly suggested by Allan J. Day). The samples of intima-media were bled and placed in preweighed vials. After removal from the animal, the arterial tissue was maintained on ice whenever possible. Within the injured areas, non-blue regions about the branch arteries represented <4% and <10% of the surface area at 3 and 5 days after injury, respectively. These small unstained regions, presumably areas of endothelial regeneration from branch arteries, were not separated from the surrounding blue field. In four of the 21 animals, a few blue streaks were found in the central or distal nonballooned areas. These blue areas, presumed to have been scratched by the tip of the deflated balloon, were separated from the remaining (white) nonballed area. The measured values of cholesteryl ester label and mass in the blue streaks within the nonballed areas were intermediate between the corresponding values in ballooned areas and the non-blue nonballed areas (data not shown). The intima-media samples were weighed and either extracted immediately or frozen at -20°C until analyzed.

Assessment of Adhering Plasma and Red Cells

In some cases, 125I-rabbit serum albumin was injected intravenously to estimate the contribution of adhering plasma to tissue radioactivity and cholesterol content. The albumin was iodinated with iodine monochloride (38 μCi/mg rabbit serum albumin)23-24 and was dialyzed against five to six changes (125 volumes) of 0.15 M saline at 4°C. At 4 to 7 minutes after intravenous injection of 125 μCi 125I per kg, each rabbit was anesthetized and a blood sample was taken by heart puncture. The arterial system was then perfused as described above. To determine the amount of plasma adhering to the tissue, radioactivity in the intima-media was divided by the terminal plasma radioactivity per ml. The values of adhering plasma obtained with labelled albumin were much larger in injured areas than in noninjured areas and were quite variable. This was probably due to a substantial uptake of albumin by the injured artery, thereby extracting much of the radioactivity associated with the injured artery. The mean blood radioactivities during the entire interval were compared with the arterial radioactivities; hematocrits were used to determine the volumes of red cells attached to the arterial tissues after one day of exposure to the labelled red cells.

Tissue and Plasma Analysis

Each intima-media sample was minced finely in a small volume of methanol and extracted with greater than 20 volumes of 2:1 (vol/vol) chloroform/methanol. After a wash with 0.2 volume of water,26 solvent was removed under nitrogen, and nonesterified and esterified cholesterol fractions in each lipid extract were separated by TLC on precoated silica gel plates developed in 50:50:1 (vol/vol) hexane/diethyl ether/acidic acid. Each fraction was eluted with chloroform/methanol (9:1, vol/vol).

Radioactivity was measured by a Beckman LS8100 liquid scintillation counter in a toluene-based scintillator containing 2% ethanol. In some cases, nonesterified and esterified cholesterol masses in the arterial samples were assayed after counting. The solvent was removed and cholesterol was estimated by the ferric chloride method of Zak et al.27 after saponification28 of the dried lipid-fluor film. The presence of the scintillators from the scintillation fluid did not affect the results of the cholesterol assay.

In most cases, the nonesterified and esterified cholesterol contents of arterial samples were measured by gas-liquid chromatography of TLC fractions. An internal standard, cholesterol methyl ether, was added to each sample and to standards containing various known amounts of nonesterified cholesterol. After saponification,28 a concentrated extract was applied to a 4-mm i.d. by 30-cm glass column packed with 3% QF-1 by weight on 100/120 Gas Chrom Q (Applied Science Laboratories, Incorporated, State College, Pennsylvania). At an oven temperature of 225°C and a carrier gas flow rate of 40 ml/minute, the retention times for the internal standard and nonesterified cholesterol were 2.2 and 3.1 minutes, respectively. Replicate determinations by means of peak height cholesterol/cholesterol methyl ether ratios gave a 0.4% coefficient of variation (n = 10). Over a range of peak height ratios from 0.3 to 2.7, a linear relationship was obtained with <2% standard error of estimate of the slope.

Plasma apolar lipids were extracted with ethanol/hexane.29 Aliquots of the extract were taken for analysis of total cholesterol, total radioactivity, and thin-layer chromatographic separation of nonesterified and esterified cholesterol in the same solvent system that was used for the arterial samples. Cholesterol mass in the total extracts, and in selected samples of nonesterified and esterified cholesterol, was determined by the method of Zak et al.27 after saponification.28 When nonesterified and esterified cholesterol mass were not needed, radioactivity was de-
Comparison between Injured and Noninjured Areas

We evaluated the effect of injury to the thoracic and abdominal aortas by analysis of variance (ANOVA) with a repeated measures design. The within-animal factors were region (thoracic and abdominal aortas) and treatment (injured and uninjured control). In some cases we compared arteries studied at different times after injury by including a grouping factor (days after injury) in our analyses. All analyses were done with Program P2V (BMDP Statistical Software; Los Angeles, California). Figure 1 shows the location of the injured and noninjured areas. The sterol content and flux tended to decrease distally along the aorta. For example, 6.5 ± 1.2, 4.7 ± 0.6, and 3.7 ± 0.4 µg cholesteryl ester/cm² in the proximal, central, and distal nonballooned areas, respectively (mean ± SEM, n = 21). Therefore, we thought that the average of the nonballooned areas above and below each injured area would give the best estimate of the sterol label or mass that would have been in that injured site had it not been injured. Thus we used the average of the proximal and central nonballooned areas as the control for the thoracic injured area, and the average of the central and distal nonballooned areas as the control for the injured abdominal aorta. When variances of measured or calculated quantities for the injured and noninjured areas differed, as they did for cholesterol content and total influx, a logarithmic transformation resulted in similar variances in the noninjured and injured areas. The thoracic and abdominal aorta responded similarly to the injury. Therefore, we averaged the injured areas in each animal and used these averages to calculate the group means which are shown in the tables. The mean values of the uninjured areas were calculated similarly.

Results

Injury

The areas of injured artery were relatively uniformly stained with Evans blue dye. In addition, hematoxylin- and eosin-stained sections of fixed arterial tissue showed few, if any, endothelial cells. However, sections stained with Verhoeff's elastic stain and counterstained with Van Gieson, as judged by light microscopy, suggested that the present method of injury did not damage the elastic laminae. Nonetheless, the media of most injured areas contained damaged and necrotic smooth muscle cells, and granulocytes were present in many cases.

Adhering Plasma and Red Cells

After 24 hours exposure to labelled red cells, the quantity of cells adhering to the artery was about 30 nI packed cells/cm², contributing negligible amounts of esterified cholesterol and little nonesterified cholesterol to the arterial tissue. The mean value in injured areas was somewhat, but not significantly, greater than that in noninjured areas. In later experiments, we sacrificed some rabbits with injured arteries just after injecting labelled red blood cells (unpublished observation). The amounts of adhering plasma calculated from these experiments were about 8 nI/cm² in both injured and noninjured areas, which are similar to previous results found in this laboratory with labelled albumin in cholesterol-fed rabbits. We used this 8 nI/cm² value for adhering plasma and made a correction corresponding to <0.6% and <0.3% of cholesteryl ester radioactivity and mass, respectively, in most injured areas. In uninjured areas, the mean corrections were 7.8% and 1.2% of cholesteryl ester radioactivity and mass, respectively.

Evidence for Efflux of Entered Cholesteryl Ester

If the rate of cholesteryl ester influx is constant and the artery acts as a sink for entering cholesteryl ester, then the influx rate calculated from labelled cholesteryl will be independent of the length of the labelling interval, the interval between administration of label, and the collection of the artery. However, if some of the entered labelled cholesteryl ester escapes from the artery, the fraction of the total entered label remaining in the artery at death will decrease with increasing length of the labelling interval. Therefore, when the artery does not act as a sink for labelled cholesteryl ester, the calculated retained influx will be lower for long labelling intervals than for shorter labelling intervals. In injured areas the retained influxes, measured during the last 2 or 4 days of life, were only 57.5 ± 1.3 and 51.0 ± 6.0 percent (mean ± SEM, p < 0.01), respectively, of those measured in the same animals during the last day. These data suggest either that the influx of cholesteryl ester increased during the last day of life, or that some cholesteryl ester label was lost from the injured artery during 2 and 4 days of exposure to label. The data shown below do not support an increase in cholesteryl ester influx with time after injury, suggesting that loss of labelled esterified cholesterol from the artery is the more likely explanation.

In uninjured areas, the rate of retained cholesteryl ester influx measured during the last 2 or 4 days of life was similar to, or greater than, that measured during the last day. This suggests that most of the cholesteryl ester label entering the uninjured artery is retained for periods of 2 to 4 days.

Total Influx and Fractional Loss

To evaluate the role of cholesteryl ester influx in the accumulation of cholesterol in an injured artery, the total influx and fractional loss were calculated. The total influx was expressed as the plasma clearance of cholesteryl ester by the artery, i.e., uptake expressed as nI of the rabbit's own plasma per unit of time per cm² or per gram of intima-media. Because entered cholesteryl ester label appeared not to have been lost from the uninjured artery, the retained influx is equivalent to the total influx.

Table 1 shows the values of total influx and fractional loss calculated from data obtained after feeding two isotopic doses of cholesterol. The experiments were initially designed to investigate possible differences in the response of the thoracic and the abdominal aorta to injury. Because these two injured areas in the same animal re-
Table 1. Total Influx and Fractional Loss of Esterified Cholesterol from Injured and Noninjured Artery

<table>
<thead>
<tr>
<th>Post-injury interval (days)</th>
<th>Labelling interval (days)</th>
<th>Plasma esterified cholesterol (mg/ml)</th>
<th>Total influx (nl/hr/cm²)</th>
<th>Fractional loss (hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2</td>
<td>8.20</td>
<td>406</td>
<td>0.081</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>4.47</td>
<td>247</td>
<td>0.070</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>6.31</td>
<td>850</td>
<td>0.046</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>11.49</td>
<td>583</td>
<td>0.069</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>6.26</td>
<td>438</td>
<td>0.076</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>3.69</td>
<td>666</td>
<td>0.078</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>4.02</td>
<td>76.9</td>
<td>0.064</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1.70</td>
<td>183</td>
<td>0.080</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>6.46</td>
<td>175</td>
<td>0.064</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>2.81</td>
<td>225</td>
<td>0.029</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>6.22</td>
<td>136</td>
<td>0.022</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>5.69</td>
<td>137</td>
<td>0.011</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>6.99</td>
<td>234</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Means ± SEM for Injured and Noninjured Areas by Time after Injury

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Total influx (nl/hr/cm²)</th>
<th>Fractional loss (hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noninjured</td>
<td>Injured</td>
<td>Noninjured</td>
</tr>
<tr>
<td>2</td>
<td>5.4 ± 1.0</td>
<td>326 ± 805</td>
</tr>
<tr>
<td>3</td>
<td>8.5 ± 1.3</td>
<td>425 ± 110†</td>
</tr>
<tr>
<td>5</td>
<td>5.5 ± 1.6</td>
<td>183 ± 27†</td>
</tr>
</tbody>
</table>

The calculations were made as described in the Appendix after correcting the tissue cholesteryl ester radioactivity for an estimated 8 nl/cm² of adhering plasma.

Days from injury to sacrifice. *The length of the longer labelling intervals; the shorter labelling intervals were always 1 day. †Non-injured areas did not show evidence for loss of entered labelled cholesteryl ester. §Plasma cholesteryl ester radioactivity. ||p < 0.002, ||p < 0.001; injured vs noninjured, ANOVA with repeated measures on logarithms of data. **p < 0.05, ***p < 0.001; value in injured greater than 0, ANOVA with repeated measures.

The values of fractional loss and total influx were calculated from arterial and plasma radioactivity data which are subject to measurement error. In addition, the accuracy of the calculated fractional loss and total influx may depend upon how near the artery is to equilibrium with plasma by the end of the two labelling intervals. Therefore, we used an empirical method to evaluate the sensitivity of the calculated fractional loss and total influx to small perturbations of the observed arterial and plasma radioactivity, one at a time. We considered ± 5% and ± 10% changes in the two values of arterial cholesteryl ester radioactivity and a random normally distributed error in plasma cholesteryl ester radioactivity (μ = 1.0, σ = 0.05) for each animal providing data in Table 1. The results (Table 2) suggest that the calculated fractional loss would be affected 2% to 3% by each 1% error in arterial cholesteryl ester radioactivity, but only about 1% by each 1% random error in plasma cholesteryl ester radioactivity.

Table 2. Parameter Values and Sensitivities

<table>
<thead>
<tr>
<th>Labelling interval</th>
<th>Parameter</th>
<th>Mean ± SEM</th>
<th>Sensitivity to changes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δt</td>
<td>Δs</td>
<td>A(Δt)†</td>
<td>A(Δs)‡</td>
</tr>
<tr>
<td>2 days 1 day k</td>
<td>0.070 ± 0.004/hr</td>
<td>2.9 ± 0.2</td>
<td>2.9 ± 0.1 1.2 ± 0.3</td>
</tr>
<tr>
<td>4 days 1 day k</td>
<td>0.022 ± 0.004/hr</td>
<td>2.0 ± 0.3</td>
<td>1.7 ± 0.1 1.2 ± 0.4</td>
</tr>
</tbody>
</table>

The experimental values were perturbed one at a time. The changes in k and l were linearly related to perturbations in A(Δt) or A(Δs) over the range -10% to 10% change. The sensitivity of k and l to errors in plasma cholesteryl ester radioactivity was evaluated by multiplying the actual values of the two cholesteryl ester labels present in plasma by random normal numbers (μ = 1, σ = 0.05) before fitting the plasma curves. Values are means ± SEM for injured aorta from nine rabbits, Δt = 2 days, or four rabbits, Δt = 4 days.

* Sensitivity = (percent change in parameter value)/percent perturbation in experimental values. †Arterial cholesteryl ester radioactivity accumulated after the longer, Δt, labelling intervals. ‡Arterial cholesteryl ester radioactivity accumulated after the shorter, Δs, labelling interval. §Plasma cholesteryl ester radioactivity.
Table 3. Esterified and Nonesterified Cholesterol Content of Plasma and Injured and Non-Injured Rabbit Aorta

<table>
<thead>
<tr>
<th>Days after Injury</th>
<th>Noninjured aorta</th>
<th>Injured aorta</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µg/cm²)</td>
<td>(µg/cm²)</td>
<td>(mg/ml)</td>
</tr>
<tr>
<td>Esterified cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6†</td>
<td>3.64 ± 0.83</td>
<td>29.4 ± 9.5§</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>6.72 ± 2.46</td>
<td>37.0 ± 14.8§</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>5.19 ± 1.00</td>
<td>45.5 ± 16.9§</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>4.45 ± 0.68</td>
<td>49.9 ± 14.5§</td>
</tr>
<tr>
<td>Nonesterified cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>23.3 ± 1.8</td>
<td>36.2 ± 4.6‡</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>30.0 ± 6.0</td>
<td>45.7 ± 8.5§</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>23.5 ± 1.5</td>
<td>41.1 ± 5.1</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>25.0 ± 1.8</td>
<td>42.3 ± 2.4‡</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.
*Number of animals. Arterial values were calculated from the average of two injured areas or two uninjured control areas per animal. †Two of these animals were fed 1% cholesterol. All other animals were fed 0.5% cholesterol.
‡p<0.02, §p<0.01, ||p<0.001, injured vs noninjured, ANOVA with repeated measures on logarithms of data.

Esterified and Nonesterified Cholesterol Content

The esterified and nonesterified cholesterol contents of both injured and uninjured areas of rabbit aorta are shown in Table 3. We assumed that the increments in esterified and nonesterified cholesterol content of the injured artery, above those in the adjacent noninjured areas, were derived from plasma. Therefore, the arterial sterol content was not only expressed in terms of µg/cm² arterial tissue, but also in terms of µl of the rabbit's own plasma per cm² artery (for example, µg arterial cholesteryl ester/cm² artery divided by µg cholesteryl ester/µl plasma).

The elevation in cholesteryl ester content in the injured areas of artery was significant as soon as 1 day after injury (2.89 ± 1.07 vs 0.41 ± 0.15 µl/cm², mean ± SEM), and was greater 5 days after injury (8.39 ± 0.90 vs 0.93 ± 0.32 µl/cm²) The increment in nonesterified cholesterol was also significant at all time points (for example, 11.0 ± 2.1 vs 7.3 ± 1.5 µl/cm² by 1 day after injury, and 24.9 ± 6.7 vs 14.8 ± 3.8 µl/cm² 5 days after injury). Figure 3 suggests that the increment in esterified and nonesterified cholesterol content of the injured areas may increase rapidly during the first 2 to 3 days after injury but then at a lower rate.

Is Cholesteryl Ester Accumulation in Injured Aorta Consistent with Calculated Influx and Loss?

Table 4 shows the final cholesteryl ester contents of the injured arteries and the pre-injury cholesteryl ester contents of these areas (estimated from the terminal cholesteryl ester contents of the adjacent uninjured areas). Also shown are the calculated terminal cholesteryl ester accumulations in injured areas. These values were calculated from the values of total influx and fractional loss. The final column shows the percent of the observed accumulation that is accounted for by the calculated influx and efflux values. Except for a few cases in which cholesteryl ester accumulation was small, total influx and fractional loss could account for the cholesteryl ester mass accumulation almost quantitatively. In addition, the ability to account for cholesteryl ester accumulation during the first 5 days after injury was similar to that for the first 2 or 3 days after injury.

In contrast, total influx and fractional loss of nonesterified cholesterol could not account for the nonesterified cholesterol accumulation in injured areas. When we calcu-

Figure 3. Sterol increment in injured areas of rabbit aorta above the adjacent uninjured control. Top: Esterified cholesterol. Bottom: Unesterified cholesterol. Increments are given in µg sterol/cm² (O---O); plasma equivalents are µl/cm² (A-----A) and are expressed as differences between injured and uninjured areas. The mean of the average increment is given in the two injured areas within each of six animals at Day 1, four animals at both Days 2 and 5, and seven animals at Day 3.
Table 4. Cholesteryl Ester Mass Accumulation In Injured Rabbit Aorta

<table>
<thead>
<tr>
<th>Post-injury interval*</th>
<th>Measured cholesteryl ester</th>
<th>Calculated cholesteryl ester at death† (μg/cm²)</th>
<th>Percent of increment accounted for§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial†</td>
<td>At death</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.32</td>
<td>33.0</td>
<td>35.8</td>
</tr>
<tr>
<td>3</td>
<td>1.05</td>
<td>15.0</td>
<td>16.4</td>
</tr>
<tr>
<td>3</td>
<td>7.60</td>
<td>118</td>
<td>120</td>
</tr>
<tr>
<td>3</td>
<td>7.91</td>
<td>96.4</td>
<td>104</td>
</tr>
<tr>
<td>3</td>
<td>7.52</td>
<td>41.5</td>
<td>43.6</td>
</tr>
<tr>
<td>3</td>
<td>2.01</td>
<td>33.8</td>
<td>32.8</td>
</tr>
<tr>
<td>3</td>
<td>2.99</td>
<td>6.21</td>
<td>7.76</td>
</tr>
<tr>
<td>5</td>
<td>3.24</td>
<td>4.72</td>
<td>6.21</td>
</tr>
<tr>
<td>3</td>
<td>5.98</td>
<td>17.9</td>
<td>23.6</td>
</tr>
<tr>
<td>5</td>
<td>5.13</td>
<td>22.9</td>
<td>26.1</td>
</tr>
<tr>
<td>5</td>
<td>5.90</td>
<td>37.1</td>
<td>41.6</td>
</tr>
<tr>
<td>5</td>
<td>2.81</td>
<td>53.3</td>
<td>54.4</td>
</tr>
<tr>
<td>5</td>
<td>3.95</td>
<td>90.4</td>
<td>83.2</td>
</tr>
</tbody>
</table>

Values are means ± SEM: 117 ± 6.
*Same as Table 1; see Table 1 also for plasma esterified cholesterol concentrations.
†Estimated from the average of the terminal cholesteryl ester mass in the two noninjured control areas.
‡For the time of death, calculated mass is (I/k)(1 - e−kt) + Initial mass. I = mean total influx, μg/hr/cm² and k = mean fractional loss, hr⁻¹, of the two injured areas within each animal.
§(Calculated final mass at the time of death + estimated initial mass)/(observed final mass - initial mass).

Relationship between Cholesteryl Ester Influx and Cholesteryl Ester Content

The daily increment in cholesteryl ester content in the injured artery was positively related (r = 0.90) to the total cholesteryl ester influx (Figure 4). A similar relationship existed between the increment in cholesteryl ester content in injured areas and the retained influx measured during the last day of life. In noninjured areas, cholesteryl ester content and total cholesteryl ester influx were also positively related (r = 0.65, Figure 5).

Discussion

The objective of our experiments was to study the influx, efflux, and accumulation of lipoprotein cholesteryl ester in deendothelialized artery prior to alterations in the artery itself due to prolonged cholesterol feeding. With short-term (14 to 17 days) cholesterol feeding, the plasma cholesterol concentrations were elevated to about 10 mg/ml. However, the esterified and nonesterified cholesterol contents of the uninjured artery, about 5 and 25 μg/cm², respectively, were similar to values reported for chow-fed rabbits.32

We used the adjacent uninjured areas of artery as control areas in order to increase sensitivity of the measurements. Although it is possible that indirect, systemic effects of injury would affect influx, we found that the total cholesteryl ester influx in the uninjured control areas, 5 to 8 nl/hr/cm², was similar to influx into arteries of rabbits fed cholesterol for 10 days but not subjected to surgery.33 Thus, it is unlikely that balloon injury affects the influx of cholesteryl ester in uninjured areas of the artery.

The method of injury used in these studies produced complete, or nearly complete, removal of the endothelium, as indicated by Evans blue staining19 and confirmed by hematoxylin- and eosin-stained sections of the injured ar-
CHOLESTERYL ESTER FLUX IN INJURED AORTA

Schwenke and Zilversmit

tery. However, we also observed some damage to medial smooth muscle cells and the presence of granulocytes in many instances. We were surprised to find medial damage, because other investigators who have balloon-injured the aortas of rabbits of similar size, using similar or greater pressures, have not reported such damage. In addition, in our experiments the balloons were inflated with liquid, which has been shown to result in lower lateral wall pressures than gas-filled balloons used by the other investigators. However, Jensen and Olin have shown that in rabbits there is a small margin between dislocation of the artery and overdistention resulting in medial damage.

Our results suggest that most of the esterified and nonesterified cholesterol that accumulated in the injured artery during the first 5 days after injury did so in the first 2 to 3 days after injury. Since cholesteryl ester influx continued, some of the cholesteryl ester entering the injured aorta must have been lost. By measuring the accumulation of radiolabelled cholesteryl ester in the injured artery during two overlapping time periods, we were able to calculate the fractional loss of labelled cholesteryl ester from the injured aorta. The values of fractional loss and total influx of cholesteryl ester calculated from radioactivity data could account for the cholesteryl ester accumulating in the same injured artery.

It was unexpected, however, that the values of fractional loss calculated in animals sacrificed 2 to 3 days after injury, 0.070 hr⁻¹, were about three times as great as those for animals sacrificed 5 days after injury, 0.022 hr⁻¹. This difference exceeds the amount of calculation error predicted by the sensitivity analysis. We were concerned that each of the calculated values for cholesteryl ester mass of the injured aorta at the time of death was close to its steady state value. That this was not an artifact of the method of calculation was determined in the following manner: We calculated arterial radioactivity from a known plasma radioactivity curve, a constant total influx, and either 0.022 or 0.070 hr⁻¹ fractional loss. We then perturbed the generated arterial radioactivity curve as described in the sensitivity analysis. Fractional losses calculated as described in the Appendix, but after perturbation of the generated data, were similar for the dual label 24 and 48 hours and for the 24- and 96-hour labelling intervals.

We also tested whether either esterification of arterial nonesterified cholesterol or hydrolysis of arterial esterified cholesterol might explain the lower fractional losses calculated from the data obtained 5 days after injury. With the SAAM program, arterial esterified and nonesterified cholesterol radioactivities were calculated for 24, 48, and 96 hours of labelling for a two-pool open system containing an additional compartment of unesterified cholesterol reversibly connected to the esterified cholesterol compartment, representing esterification and hydrolysis of arterial sterol fractions. Even over a wide range of assumed rate constants, fractional loss calculated for 24 and 48 hours and 24 and 96 hours dual-labelling intervals were similar. Therefore, we suggest that the lower fractional loss in the animals studied 5 days after injury represents a biological event rather than an artifact due to an oversimplistic kinetic model.

A change in fractional loss would be consistent with reparative changes in the aorta. During the first 2 to 3 days after injury, the subendothelium is partially covered by platelets and leukocytes. The present data indicate that the calculated fractional loss and total influx were similar during this interval (Table 1). Beginning on the third day after injury, Spaet et al. found occasional neointimal smooth muscle cells on the subendothelium of the injured aorta. The smooth muscle cell cover was greater on the fourth day and was nearly complete by the fifth day after injury. Between the third and the sixth days after injury, the chondroitin sulfate content of the injured aorta increased about 40%. In vitro, chondroitin sulfate has a high affinity for very low and low density lipoprotein, and complexes containing lipoprotein and chondroitin sulfate can be extracted from the aortas of hypercholesterolemic rabbits.

Increased binding of enterolipoprotein cholesteryl ester by greater amounts of chondroitin sulfate in the injured aorta, and the uptake and retention of some of the entered lipoprotein cholesteryl ester by the proliferating smooth muscle cells during the third to the fifth days after injury, which would be predicted by experiments in vitro, would be consistent with the lower value of fractional loss calculated for the second through fifth days after injury.

During the first 5 days after injury, cholesteryl ester entered the injured artery at a high rate. We calculated the total cholesteryl ester influx to be 4.39 μg/day/cm² in aorta injured 5 days previously. This value is similar to the 5.72 μg/day/cm² cholesteryl ester influx reported by Day et al. in hypercholesterolemic rabbits for deendothelialized areas of aorta that had been allowed to heal for 15 weeks after injury.

Our report proposes that cholesteryl ester influx, presumably reflecting lipoprotein influx from plasma, could account for the observed accumulation of cholesteryl ester in the injured artery. Our data suggest that differences in total influx might explain a large portion of the variability in the amount of cholesteryl ester accumulation in injured aortas (Figure 4). In addition, we also considered other sources of esterified and nonesterified cholesterol in the injured artery. One possible source of nonesterified cholesterol in the injured aorta is from cells that enter or adhere to the artery. Several investigators have reported that a monolayer of platelets covers the injured aorta within 30 minutes of the injury and that this layer remains relatively unchanged for 24 hours, but is nearly gone 7 days after injury. However, if the number of platelets adhering 30 minutes after injury equals approximately 4.2 × 10⁶/cm², and if we use the cholesterol content of platelets from cholesterol-fed guinea pigs, then platelets could account for only about 0.3 μg nonesterified cholesterol/cm² injured artery, about 1% of the observed accumulations, and for almost no esterified cholesterol. Our experiments with labelled red cells injected just before or just after injury indicated that the amount of red cells adhering to the injured artery 1 day after injury would be no more than 30 nl/cm². Using a value of erythrocyte cholesterol reported for rabbits fed cholesterol for 15 days, we calculated that red cells could contribute at most about 0.8 μg nonesterified cholesterol/cm² injured artery, 2% to 3% of the observed accumulations, and again almost no esterified cholesterol.
Relatively fewer white cells than platelets have been observed adhering to the injured artery. Therefore, it appears that cellular elements adhering to or entering the injured aorta cannot account for the large amount of esterified and nonesterified cholesterol present in the injured artery during the first 5 days after injury.

Our results suggest that esterified and nonesterified cholesterol in the injured artery increased rapidly during the first 2 to 3 days after injury and then at a slower rate. At 5 days after injury, our value of cholesteryl ester accumulation in the injured areas, 2.49 mg/g wet weight, was slightly less than that reported by Katocs et al.47 for hypercholesterolemic rabbits 7 days after injury, 3.52 mg/g wet weight (mean for thoracic and abdominal aorta). However, both of these values are much lower than the 42 to 64 mg/g (66% to 69% ester) and 25 to 28 mg/g (74% ester) total cholesterol reported for reendothelialized and still deendothelialized aorta, respectively, after 6 to 10 weeks of hypercholesterolemia and 11 to 15 weeks after balloon injury.3 The accumulation of cholesteryl ester beyond the amounts predicted from total influx and fractional loss of cholesteryl ester calculated during the first 5 days after injury could at least partially be due to retention of entering cholesteryl ester by glycosaminoglycan42 and collagen43 that accumulate after injury.13, 49

In summary, we have shown: 1) total cholesteryl ester influx in acutely injured artery is high, 2) these injured areas experience a high turnover of cholesteryl ester, and 3) cholesteryl ester accumulates in injured areas despite the high rates of loss.

Acknowledgments

The authors gratefully acknowledge the skillful technical assistance of Lorraine Maleckl and the aid of Joan Roberts in preparing the manuscript. We also thank S. Klumpp for microscopic evaluation of the extent of arterial injury.

References

33. Stender S, Christensen S, Nyvad O. Uptake of labelled free and esterified cholesterol from plasma by the aortic intima-
media tissue measured in vivo in three animal species. Atherosclerosis 1978;31:279-293


Appendix

The labile arterial cholesteryl ester pool is described by the equation:

\[
dA(t)/dt = IP(t) - kA(t)
\]

A(t) = arterial cholesteryl ester radioactivity at time t, dpm/cm²; I = total influx of cholesteryl ester (or clearance of plasma cholesteryl ester by the artery), nl/hr/cm²; P(t) = plasma cholesteryl ester radioactivity at time t, dpm/nl; k = fractional loss of cholesteryl ester from the artery, hr⁻¹.

The plasma cholesteryl ester radioactivity curves after oral doses of labelled cholesterol were well described by polynomials. Third or fourth degree polynomials worked well for plasma curves followed for 1 to 2 days. However, fifth or sixth degree equations were frequently used to fit curves followed for 4 days as shown in Figure 2. If the equation for the plasma cholesteryl ester radioactivity:

\[
P(t) = c_0 + c_1t + \ldots + c_nt^n (c_0, c_1, \ldots, c_n \text{ constant})
\]

is substituted into Equation 1, the resulting equation can be solved to obtain:

\[
A(t) = \sum \left[ \frac{c_0 n!}{k} \left( \frac{e^{-kt} - 1}{(-kt)^n} \right) + \frac{c_1 n!}{(-kt)^{n+1}} \left( \frac{e^{-kt} - (-kt)^n}{n!} \right) \ldots + \right] \left[ \frac{(-kt)^{n-1}}{n!} - \frac{(-kt)^n}{(n-1)!} \right]
\]

If an animal is given two doses of label and sacrificed at a time which is \( \Delta t_1 \) hours (the longer interval) after administration of the first label and \( \Delta t_2 \) hours (the shorter interval) after dosing with the second label, Equation 3 can be written for each isotope. The ratio of Equation 3 written for time \( \Delta t_1 \) to that for time \( \Delta t_2 \) contains only the known values of \( c_i \) (i = 1 to n) derived from the plasma curves (a different set for each label) and a single unknown, the fractional loss, k. The value of k can be found by iteration and, when substituted into Equation 3, results in the total influx I.
Enhanced accumulation and turnover of esterified cholesterol in injured rabbit aorta.
D C Schwenke and D B Zilversmit

doi: 10.1161/01.ATV.7.4.367

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/7/4/367

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/