In Vitro Synthesis of DNA, Protein, and Lipids by the De-endothelialized Rabbit Aorta

Robert S. Rosenfeld, Ludovic Drouet, Jose Cintron, Inge Paul, Jorge Won, and Theodore H. Spaet

The uptake of \(^{3}H\) leucine, \(^{3}H\) thymidine, \(^{14}C\) acetate, and \(^{14}C\) mevalonic acid by aortic intima media from normal rabbits and from rabbits subjected to a single balloon de-endothelialization was measured 6 days, 2 months, and 4 months after treatment to determine how long the injury-induced stimulation of incorporation of these precursors into tissue components persisted beyond 6 days, the time of maximum proliferative response of the smooth muscle cells. We found that \(^{3}H\) thymidine incorporation (indicative of DNA synthesis and the potential for cell proliferation) was about three times greater in the de-endothelialized tissue than in the control tissue 6 days after vessel injury, but that by 2 months it was normal. Labeled leucine incorporation into the de-endothelialized tissue (a measure of protein synthesis) was eight times higher than normal at the time of maximum proliferative response to injury (6 days), and showed no decrease under identical incubation conditions in balloonized tissue obtained 2 months after de-endothelialization; it continued high at 4 months. Both \(^{14}C\) acetate and \(^{14}C\) mevalonate uptake into lipids by the aortic tissue were enhanced by the injury; this increased incorporation was still evident at 4 months. Thus, de-endothelialization of the aorta triggers a vessel wall response characterized by augmented protein synthesis and lipogenesis, which persists at least 4 months after injury and at least 2 months after DNA synthesis has normalized.


In experimental animals, loss of endothelial cells from the luminal surface of the aorta due to hemodynamic, immunological, bacterial, metabolic, or mechanical factors is associated with a proliferative response, which often results in a lesion at the site of de-endothelialization. In most cases the sequence of events is similar: platelet adherence to the exposed area; presumed release of a mitogenic factor by the platelets; a response by the underlying medial smooth muscle cells (SMC) involving migration into and proliferation within the intima above the internal elastic lamina; formation of a multilayered neo-intima of SMC; and re-endothelialization over the affected area. To study lesion formation in the rabbit in a systematic way, several investigators have induced mechanical injury to the aorta by removing the endothelial cell layer by balloon catheterization. One objective has been to produce vascular damage, which ordinarily might take much longer to develop and which may have relevance to atherogenesis in humans.

Morphologic and radioautographic studies indicate that maximum migration and proliferation of SMC responding to endothelial damage of the aorta takes place within the first week after injury. In the balloonosed vessel, proliferation of intimal SMC seems to cease in about 2 months. Four months after injury, about half the luminal surface is re-endothelialized, and by 1 year, restoration of the endothelial cover over the neo-intima is nearly complete.

We would expect this injury-induced response of SMC to be accompanied by increased synthesis of DNA, protein, and lipid. Although these biosynthetic events should return to near base-
Animals

New Zealand male rabbits about 12 weeks old and weighing 2.5 kg, maintained on Purina rabbit chow, were used in all studies. The animals were acclimated to our animal room for at least 1 week before experimentation.

De-endothelialization of the Aorta

The balloon catheter technique used in this laboratory is a modification of the Baumgartner procedure. \(^{21}\) Briefly, after light pentobarbital anesthesia supplemented with ether, the femoral artery was exposed and a 4F Fogarty embolectomy catheter was passed up into the aorta via the iliac artery to the level of the aortic arch. The balloon was then inflated to a pressure of about 600 mm Hg and pulled back, while inflated, to the iliac artery. This procedure, taking about 15 seconds, was repeated twice and the catheter was rotated each time to compensate for possible asymmetry and to ensure complete removal of the endothelium. The balloon was then collapsed, the catheter withdrawn, the femoral artery double ligated, and the incision closed.

Preparation of Tissue

One hour before sacrifice, 1 ml of Evan’s blue dye was injected into an ear vein. The dye stains the luminal surface area of the vessel lacking endothelial cover and shows the extent of re-endothelialization of the denuded aorta. Rabbits were sacrificed by sodium pentobarbital injection; the aorta was immediately removed and immersed in ice-cold Dulbecco’s medium where the adventitia was stripped from the vessel. The medium was changed several times during the procedure. Although we could not perform a test on the tissue to be incubated that would definitively document complete stripping of the adventitia, we carried out the procedure under magnification and observed no adventitia adhering to the outer media. Samples were prepared in a technique identical to that used in other studies, and light microscopy media showed no adventitia remaining on the stripped aorta. The aorta was transferred to fresh medium and cut perpendicular to the axis into 100–120 segments about one millimeter long. The rings were washed again with Dulbecco’s medium before incubation with labeled substrate.

Incubations

The procedure was similar to that described by Avigan et al. \(^{22}\) Equal portions of thoracic and abdominal aortic segments were combined for each incubation to minimize possible differences between metabolic activity of these sections. \(^{23}\) One rabbit usually provided material for 12 incubations, each containing 10 to 15 rings. Incubations were carried out in 2 ml of Krebs-Ringer bicarbonate buffer at 37°C for specified times under a 95% air-5% CO\(_2\) atmosphere. The tracers, added in the buffer, were \([1-14C]\) acetate and \([14C]\) mevalonate into sterol, \([3H]\) leucine into protein, and \([3H]\) thymidine into DNA in aortic tissue taken from rabbits subjected to a single balloon de-endothelialization 2 years before sacrifice, \(^{8}\) as well as furnish substrate for cholesterol esterification in neo-intimal cells. \(^{16-20}\) We now report studies on the incorporation of crystalline cholesterol and cell debris observed in the aortas of rabbits subjected to a single balloon de-endothelialization 2 years before sacrifice, \(^{8}\) as well as furnish substrate for cholesterol esterification in neo-intimal cells. \(^{16-20}\) Such processes, along with the uptake of plasma lipoprotein cholesterol, might be associated with the accumulation of crystalline cholesterol and cell debris observed in the aortas of rabbits subjected to a single balloon de-endothelialization 2 years before sacrifice, \(^{8}\) as well as furnish substrate for cholesterol esterification in neo-intimal cells. \(^{16-20}\) Although we could not perform a test on the tissue to be incubated that would definitively document complete stripping of the adventitia, we carried out the procedure under magnification and observed no adventitia adhering to the outer media. Samples were prepared in a technique identical to that used in other studies, and light microscopy media showed no adventitia remaining on the stripped aorta. The aorta was transferred to fresh medium and cut perpendicular to the axis into 100–120 segments about one millimeter long. The rings were washed again with Dulbecco’s medium before incubation with labeled substrate.

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Extraction, Isolation, and Counting Procedures

The 20% TCA solution was removed from the segments and the tissue washed once with 5 ml
obtained by successive extractions of the tissue portions of 5% TCA and 0.9% NaCl. Lipids were obtained by successive extractions of the tissue with 5 ml hot acetone:ethanol (1:1); then twice at room temperature with 5 ml acetone:ether (1:1); and, finally, three times with 5 ml ether. These washings were combined and concentrated in vacuo to afford the total lipid extract. The tissue residue was then washed with 5% TCA, with 0.9% NaCl, twice with methanol, and with ether. These last washings were discarded, and the tissue residue was air dried and weighed.

Before saponification of the lipid extract, tracer quantities of [3H] cholesterol were added for recovery estimation. After refluxing for 3 hours in 10% sodium hydroxide in 80% ethanol, the reaction mixture was extracted with petroleum ether to obtain the nonsaponifiable portion. This fraction was chromatographed on a 20 X 20 cm plate coated with a 0.25 mm layer of silica gel in the system, ethyl acetate:cyclohexane (3:7). Bands corresponding to the position of cholesterol were scraped from the plates and extracted with chloroform:methanol (2:1). An aliquot of each extract was counted, cholesterol recovery being determined from the tritium content.

All counting was carried out in a Tracer Analytic liquid scintillation spectrometer, Model 6892. After extraction from the aortic tissue, lipids and sterols contained only 14C; the 3H derived from [3H] leucine was negligible24 so that [3H] cholesterol could be used for a recovery standard, and the fractions were counted in the double-labeled mode.25 Tissue residues were solubilized in perchloric acid-hydrogen peroxide mixture before the addition of scintillant, and counted in the double-labeled mode, since both [14C] acetate and [3H] leucine are incorporated into protein. Where [3H] thymidine was incubated with the tissue, the 3H in the tissue residue represented incorporation into DNA. Appropriate quench corrections were included. Data are expressed as cpm per mg of dry tissue since we found that expressing them in relation to DNA content gave no information not available from basing the radioactivity on dry weight of tissue. For example, in controls, we found 5.1 ± 0.26 (SD) µg DNA/milligram of dry tissue in the aortic segments. Animals that had been balloon 30 days earlier showed 5.3 ± 0.57 µg/mg. DNA assay of segments from rabbits sacrificed 60 days after de-endothelialization showed 4.18 ± 0.61 µg/mg as compared with 4.21 ± 0.98 µg/mg in controls. Studies in progress indicate that the DNA content of aorta 1 year after balloon de-endothelialization does not differ from controls. An increased number of cells per mass of tissue should show an increased DNA content; we did not find this either 1 or 2 months after balloon injury. Other workers in this area have also reported their data in "per milligram of tissue."22, 27

Results

Part 1

Five rabbits were subjected to single balloon catheterization 60 days before sacrifice, and segments of aortic media obtained at sacrifice were incubated as described for 2, 4, or 8 hours. Four rabbits maintained under the same conditions but not subjected to surgery served as controls; their aortic tissue was treated identically. Areas free of endothelial cover and stained blue comprised about 50% of the surface area; white areas, where re-endothelialization had taken place, were present as oval patches surrounding branch orifices, conforming to earlier observations.28 Neo-intimal growth in response to injury was apparent, as the total average weight of the dried aortic segments in ballooned animals (35.8 mg/kg body weight ± 3.18 SD) was significantly greater than that in controls (22.3 ± 2.58 mg/kg body weight — p < 0.001). As judged by the continued incorporation of labeled leucine, thymidine, and acetate or mevalonate into protein, DNA, and lipids, respectively, the aortic segments from both experimental and control rabbits remained metabolically active throughout the period of observation, up to 8 hours of incubation (figure 1).

Incorporation of [3H] Thymidine

Tritium remaining in the dried tissue residue after incubation of aortic media segments with [3H] thymidine is a measure of DNA synthesis. Table 1 shows that there is no statistical difference between the incorporation of [3H] thymidine into aortic tissue after the 4- and 8-hour incubations in the two groups of rabbits. This confirms earlier findings29 that by 2 months the proliferative activity of the ballooned aortic segments has returned to that of nonballooned tissue.

Incorporation of [3H] Leucine and [14C] Acetate into Lipid Extracted Tissue Residue

Figure 1 shows the incorporation of [3H] leucine, a measure of protein synthesis, into tissue residue. This figure and table 1 show that the media from de-endothelialized vessels incorporates approximately six-, eight-, and 12-fold more radioactivity at 2, 4, and 8 hours of incubation, respectively, as compared with incubated segments from nonballooned controls (p < 0.01). Uptake of 14C into the same tissue samples from [14C] acetate was also greater in the de-endothelialized segments, giving results almost identical to those from [3H] leucine (p < 0.01, figure 1) since acetate can be readily incorporated into nonlipid as well as lipid constituents.
Incorporation of \([{}^{14}\text{C}]\) Acetate and \([{}^{14}\text{C}]\) MVA into Nonsaponifiable Lipids and Cholesterol

Despite considerable variability among the five ballooned and four control rabbits, there was a two- to fourfold increase of \([{}^{14}\text{C}]\) acetate incorporation into the nonsaponifiable fraction, and a three- to sixfold increase into the cholesterol in the de-endothelialized tissue, indicating a persistent stimulation of sterol synthesis (Table 1). Removal of the aortic endothelium 2 months before sacrifice was associated with greater incorporation of \([{}^{14}\text{C}]\) mevalonic acid (MVA) into both the nonsaponifiable fraction and cholesterol (\(p <\))
very little in appearance from the 2-month balloon
catheterization. The experimental design was identical to that of the
four controls, were subjected to single balloon
catheterization 120 days before sacrifice. The
error represents the standard error of the ratio of two means
compared to four controls.

*Data represent the average ratio of incorporation of radioactivity into aortic segments from ballooned rabbits
compared to nonballooned rabbits.

The ratios were obtained by dividing the average cpm/mg tissue (ballooned) by the corresponding average for
the controls, which were taken from figure 1. The error represents the standard error of the ratio of two means
p 232). The figures in parentheses show ranges. In the first group ballooned 6 days before sacrifice, three rabbits
were compared to a single control. In the second group ballooned 60 days before sacrifice, five rabbits were
compared to four controls. In the third group ballooned 120 days before sacrifice, four rabbits were compared to
close control segments.

Abbreviations: PB = postballooning; Leu = leucine; Ac = Acetate; Thym = thymidine; MVA = mevalonate.

As judged by the incorporation of labeled precursors, the metabolic activity of the aortic segments 4 months after de-endothelialization was remarkably similar to that of the 2-month study
(figure 1 and table 1). There was also no significant
difference between the uptake of [3H] thymi-
dine in the experimental and control tissues after
4 or 8 hours. Incorporation of [3H] leucine and
[14C] acetate into protein and nonlipid tissue con-
stituents remained elevated in the segments from
the ballooned rabbits as compared to the con-
trols (p < 0.01). Labeled acetate and mevalonate
were taken up in greater amounts for nonsaponi-
fiable lipid and cholesterol biosynthesis (p < 0.01).

We could not definitely determine whether there were any differences in the metabolic activity
between the segments from the 4- and 2-
month balloononed animals. While there was less incorporation of [3H] leucine and
more uptake of [14C] acetate into nonsaponi-
fiable lipids in the 4-month tissue, the differences

0.02) after 8 hours incubation of the segments as
compared to the controls. Use of [14C] MVA for
sterol and cholesterol biosynthesis was less
affected by the experimental procedure than was
the incorporation of [14C] acetate, presumably
because the 6-carbon precursor is beyond the
hydroxymethylglutaryl co-enzyme A reductase
step, usually considered to be rate controlling.

Part 2

A group of eight rabbits, four ballooned and
four controls, were subjected to single balloon
catheterization 120 days before sacrifice. The
experimental design was identical to that of the
2-month study. Visual inspection by three observ-
ers showed that the de-endothelialized rabbit
aortas that were stripped of adventitia differed
very little in appearance from the 2-month bal-
looned aortas. There was, however, increased re-
endothelialization of the luminal surface, as evi-
denced by areas of Evan's blue dye exclusion.
were not significant \((p > 0.05)\). Possibly, differences in metabolic activity of the aortic rings at 2 and 4 months after vessel injury were too small to be detected in this experiment; however, balloononed aortic tissue showed increased sterologenesis from both \(^{14}\text{C}\) labeled precursors even 4 months after injury.

**Part 3**

To obtain data on the metabolic activity of the aortic segments at the time of maximum proliferative response to de-endothelialization, about 6 days after injury,\(^a,26\) three rabbits were subjected to a single balloon catheterization, and sacrificed 6 days later. These rabbits and one control rabbit were treated as described, and the results are shown in table 1. Because of the small number of animals, the standard deviations were not calculated, and the ranges are given with each average. Table 2 presents the data for these rabbits in cpm/mg dry tissue. The incorporation of \(^3\text{H}\) leucine into protein as well as \(^{14}\text{C}\) acetate and \(^{14}\text{C}\) MVA into nonsaponifiable lipid and cholesterol are remarkably similar at 6, 60, and 120 days after balloononing. Only the incorporation of \(^3\text{H}\) thymidine into DNA was substantially different at 6 days where it was about three times higher than in segments from nonballooned aorta. It is interesting that at 2 months and 4 months after balloononing, when \(^3\text{H}\) thymidine incorporation had subsided to control levels, protein and lipid synthesis persisted at levels above control values (table 1).

**Discussion**

The results of this study demonstrate that sterol and protein synthesis in the injured aorta continue at an elevated rate long after mitotic activity has subsided to baseline levels, which is consistent with earlier reports from this laboratory based upon radioautographic and morphometric evidence.\(^9,12\) Although some studies show that the major proportions of aortic cholesterol and its esters are derived from circulating low density lipoproteins (LDL) which enter the cells by receptor mechanisms\(^30\) and nonreceptor inclusion processes,\(^31\) and that the severity of aortic atherosclerosis in animal models is directly related to circulating cholesterol levels,\(^32\) there is also abundant evidence that sterol synthesis takes place in arterial tissue. Both aortic smooth muscle cells and endothelial cells synthesize cholesterol, with the former showing greater activity.\(^32,33\) Avigan et al.\(^25\) found that aortic strips and minced aortic tissue from rabbits converted \(^{14}\text{H}\) acetate to cholesterol, and they and others showed the process to occur in SMC from aortas of several experimental animals, where control was exerted at the formation of mevalonic acid from HMG-CoA.\(^34,35\) It has been suggested that some in situ cholesterogenesis may be necessary for the elaboration and maintenance of membrane structures in growing or proliferating cells, even in the presence of lipoprotein-bound cholesterol supplied from the culture medium.\(^13,14\)

Evidence of increased and persistent cholesterol biosynthesis by the de-endothelialized aorta is suggested by the work of Spaet et al.\(^8\) who reported the development of lipid-laden aortic plaques containing cholesterol crystals in rabbits that had been de-endothelialized 2 years earlier and had been fed a normal, nonatherogenic diet throughout the period. These observations are not inconsistent with the investigations of Minick et al.,\(^28\) Falcone et al.,\(^27\) and Moore,\(^10\) who observed that, in rabbits, intimal lipid deposits consisting largely of cholesterol and its esters were present in larger quantities under areas of regenerated endothelial cover than in the still denuded areas where the SMC of the neo-intima are in direct contact with circulating blood. Although they are cautious in interpreting their data, these investigators do not believe that the lipid accumulation in the affected areas is solely the consequence of passive transport of lipopro-
tein into the arterial wall. Although rabbits with experimentally induced aortic lesions show up
take of cholesterol from the plasma by the les-
ion,\(^\text{16, 30}\) Day et al.\(^\text{18}\) implied that plaque choles-
sterol had an origin other than plasma. They
demonstrated that the concentrations of free and
ester cholesterol were greater in the lesions than
in the normal aortic intima, appearing rapidly
within the plaques despite low levels of circulat-
ing cholesterol.

In the present studies, the vessel segments
showed, 2 months after aortic de-endothelializa-
tion, a perturbed pattern of lipid and protein
metabolism as compared to unballooned tissue.
Ballooned arterial segments placed in the incuba-
tion medium immediately after excision, rinsed,
with fat and adventitia removed, incorporated
more radioactivity from \([\text{3H}]\) leucine and \([\text{14C}]\)
acetate into protein, and more radioactivity from
\([\text{14C}]\) mevalonate into lipids and sterols than did
nonballooned tissue. Note that this system is an
in vitro incubation and does not have the charac-
teristics of an explant since the necessary condi-
tions and additives are lacking; thus, no changes
in cellular morphology would be expected to
occur under our conditions. However, \([\text{3H}]\) thymi-
dine uptake (indicative of DNA synthesis and cel-
lar proliferation) was not significantly different
from that in the controls (table 1). This was in
marked contrast to \([\text{3H}]\) thymidine incorporation at the
time of maximal response to aortic de-
endothelialization, 6 days after the procedure,
when SMC were migrating into and rapidly prolif-
erating above the internal elastic lamina\(^\text{29}\) and
when \([\text{3H}]\) thymidine uptake was shown to be
threefold higher than in the controls (table 1). If
the tissue incubations relate to the in vivo situ-
ton, then sometime before 60 days after balloon
catheterization of the aorta the augmented cel-
ular proliferation had apparently subsided, but
increased protein and lipid synthesis continued.
Since the data are expressed as cpm/mg of tis-
sue, the results suggest enhanced metabolic
activity of the neo-intimal and medial cells of the
ballooned aorta.

Conceivably, damage to cells caused by sec-
tioning the aorta into small rings could stimulate,
or perhaps suppress, the metabolic processes
under study. However, since we treated both con-
trol and experimental vessels identically, pertur-
bations introduced by these procedures should
produce similar effects.

It is unlikely that the differences in pool size of
the labeled precursors between control and ex-
perimental tissues could account for the stimula-
tion observed in the latter, for several reasons.
1) After tissue segment preparation, which is car-
ried out in several changes of buffer before incu-
bation, endogenous pools of the precursors are
probably minimal in both control and experimen-
tal segments. Further, the supply of labeled pre-
cursor appears to be adequate; the concen-
trations of radioactivity in the Krebs-Ringer
bicarbonate buffer were essentially unchanged
after incubation and were manyfold higher than
the amounts incorporated into tissue constitu-
ants. 2) Results from the incorporation of the
radioactive precursors into tissue constituents of
ballooned aortas are in accord with morpho-
metric observations.\(^\text{9, 12}\) 3) Incorporation of \([\text{3H}]\)
leucine and \([\text{14C}]\) acetate into tissue proteins by aortic
segments from de-endothelialized rabbits always
shows the same increase when compared
with controls, \(p < 0.01\).

Although the standard deviations are large,
probably due to animal variability and to smooth
muscle cell damage associated with ballooning
procedures, the differences between control and
ballooned vessels are large enough to be highly
significant. In the aortic tissue from both experi-
mental and control animals, incorporation of
labeled precursors into tissue constituents gen-
erally increased with time, indicating that no essen-
tial endogenous factor had become depleted dur-
ing incubations and suggesting that future
studies might concentrate on the 8-hour incuba-
tion period where the greatest differences were
observed.

It is possible that our results may indicate nor-
mal metabolic activity for the cells and that the
increases in incorporation of the labeled pre-
cursors may reflect the changed number and con-
centration of the cells in the segments from de-
endothelialized aorta. This is unlikely since,
assuming that cells in ballooned tissue have the
same metabolic activity as controls, about 10
times more cells per mg of dry tissue in the bal-
looned segments would be required to account
for the increased leucine incorporation, and
about two to five times more would be needed
to account for increased acetate and MVA incorpo-
ration. However, uptake of labeled thymidine
into DNA (an indicator of cellular proliferation)
and DNA concentration are essentially the same
in the experimental and the control aortic rings.
Thus, a more reasonable explanation assumes
increased metabolic activity, although a defini-
tive answer must await further experiments that
extend the interval between ballooning and sacri-
ifice and compare the morphology of the experi-
mental and control tissue.

These findings of increased uptake of labeled
leucine, acetate, and mevalonate by the seg-
ments in response to de-endothelialization,
coupled with the greater mass of aortic intima
media tissue, suggest that newly synthesized tis-
sue possesses both a higher rate and a greater
amount of incorporation of these protein and
lipid precursors than does control tissue. Morpho-
metric observations show that aorta that is
de-endothelialized 2 months before sacrifice has
a thicker, but stable, layer of neo-intimal cells
above the original internal elastic lamina.\(^\text{28}\)

Since aortic tissue obtained from rabbits sub-
jected to balloon de-endothelialization 4 months before sacrifice shows marked and unabated increase in protein synthesis and a modest, but significant, increase in sterol synthesis apparently related to the injury, the question arises as to the nature of the stimulation. It is possible that increased [3H] leucine uptake represents incorporation into the polypeptides of proteoglycans which reportedly increase in the neo-intima of aorta from balloononed rabbits, particularly in the re-endothelialized areas.27, 28 Although glycosaminoglycans can bind LDL,27 resulting in increased neo-intimal lipid, it has also been suggested that proteoglycans may cover lipoprotein binding sites on neo-intimal SMC.10 This would prevent internalization and lipolysis and would stimulate intracellular sterol synthesis. Our data support the latter view.

Hajjar et al.29 showed that there was no difference between de-endothelialized (blue) and re-endothelialized (white) areas of rabbit aorta in the metabolic activity of acyl cholesterol acyl transferase or of a series of intracellular marker enzymes, but they did report a greater acid cholesterol esterase in the blue area. In a different experimental model, Fritz et al.30 found no differences in DNA and protein synthesis between "flat lesions" and "nonlesion" areas of the abdominal aorta of pigs subjected to aortic de-endothelialization and maintained on an atherogenic diet; they also reported an increase in cholesterol content in the flat lesions as compared with nonlesion areas, but did not measure these parameters in nonballoned animals. Although the present investigation shows that 4 months after de-endothelialization elevated lipid and protein synthesis have not subsided, it is not known how long increased metabolic activity persists. The effects of the presence or absence of endothelial cover on the incorporation of lipid, protein, and DNA precursors by intima media tissue of balloononed aorta, and the duration and interrelation of these biosynthetic processes will provide a basis for future work.

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Index Terms: aorta, response to injury • lipid synthesis by aorta, response to injury • de-endothelialization of aorta • protein synthesis by aorta, response to injury

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