Increased Aortic DNA Synthesis Precedes Renal Hypertension in Rats
An Obligatory Step?
ALEX L. LOEB, H. GEORGE MANDEL, JAMES A. STRAW, AND BARBARA L. BEAN

SUMMARY  The rate of DNA synthesis was determined in rats with developing and established two-kidney, one clip renal hypertension. Rate of DNA synthesis was measured as [3H]thymidine incorporation into DNA per hour. After stenosis of the renal artery, blood pressure increased over a 2-week period. Five days after clipping, there was an increase in the rate of aortic DNA synthesis before an increase in blood pressure was detected, whereas there was no DNA effect in sham-operated animals. This difference in [3H]thymidine incorporation into aortic DNA could not be accounted for by alterations in thymidine pool sizes. The increase in DNA synthesis was still present 3 weeks after renal artery stenosis, although at that time blood pressure had plateaued. The role of DNA synthesis in the development of renal hypertension was investigated by determining whether inhibition of DNA synthesis with cytosine arabinoside could prevent the increase in blood pressure. Treatment of clipped rats with cytosine arabinoside for 5 days delayed the increase in blood pressure for more than 4 days, as compared with the effect of saline treatment in clipped rats. Although the possibility remains that some effect of cytosine arabinoside other than its effect on DNA synthesis could have influenced blood pressure, there were no differences in body weight, food intake, water intake, or urine output between cytosine arabinoside–treated and saline-treated rats with renal artery clips, and cytosine arabinoside treatment had no effect on blood pressure or body weight in normal rats. These results suggest that an increase in DNA synthesis may be an obligatory step in the genesis of renal hypertension.
(Hypertension 8: 754-761, 1986)

KEY WORDS  • two-kidney, one clip renal hypertension  • DNA synthesis  • vascular smooth muscle  • cytosine arabinoside

ALTHOUGH the pathogenesis of most clinical cases of arterial hypertension is unknown, some of the sequelae are always the same. Patients exhibit a marked thickening of the arteries and resistance vessels and a hyperreactivity to pressor agents. The same types of changes have been observed in animal models of hypertension and are characterized by increases in the size and number of arterial smooth muscle cells. Vascular morphology changes whether the hypertension is experimentally induced or genetically determined, as in the spontaneously hypertensive rat.

The possible factors responsible for the structural arterial changes seen in hypertension are as yet unknown; however, it appears that thickening of the vessel wall may be secondary to the development of increased blood pressure. In experimental hypertension, the thickening of the blood vessel wall is associated with increased arterial smooth muscle DNA synthesis and increased vascular wall protein synthesis.

To examine whether the structural changes seen in hypertension are caused by the pressure increase or whether the arterial morphology contributes to the elevated pressure, Lundgren et al. quantified the rate at which structural changes occurred in renal hypertension and correlated these changes with the increases in arterial blood pressure. They found that after stenosis...
of the renal artery, the blood pressure rise took about 1 week to reach its final, sustained level. Although structural changes were evident at 1 week, they were not fully developed until 2 to 3 weeks after the renal artery had been clipped. Thus, Lundgren et al.\(^1\) proposed that the vascular changes were caused by the pressure increase, since the time course of structural changes lagged considerably behind that for the change in blood pressure. This may not have been the case, however, since some structural changes were already apparent at the time of the blood pressure increase. Thus, since the Poiseuille relationship states that even a small decrease in vascular diameter can cause a large increase in resistance, the small structural changes might have preceded and induced the pressure increase. In the perfused rat mesenteric artery preparation, during the developmental phase of renal hypertension, Collis and Alps\(^3\) found that, while functional evidence of vascular thickening (i.e., an increase in vascular reactivity to pressor agents) paralleled the increase in arterial pressure, the alteration in the ratio between maximum pressor responses to norepinephrine in hypertensive and control rats lagged behind the development of hypertension by 2 weeks.

Our studies were designed in an attempt to clarify the relationship between the initiation of structural changes as measured by an increase in aortic DNA synthesis and the rise in blood pressure in two-kidney one clip renal hypertension. In addition, the role of DNA synthesis during the initial stages of renal hypertension was investigated using the DNA synthesis inhibitor cytosine arabinoside (Ara-C) to block DNA synthesis during the initial stages of renal hypertension.

**Materials and Methods**

Female Wistar rats weighing 190 to 250 g (Harlan Corp., Frederick, MD, USA) were housed in groups of four to six in wire mesh cages in rooms with a 12-hour light/dark cycle. They were fed Purina Rat Chow (Ralston-Purina, St. Louis, MO, USA) and tap water ad libitum.

Experimental renal hypertension was induced by placing a 0.2-mm silver clip on the left renal artery, leaving the contralateral kidney untouched. Rats were anesthetized with ether, and the abdomen was shaved before operation. An incision was made from the pubis to xiphisternum, through both skin and body wall. The gut was displaced to the side and wrapped in moist, sterile gauze. The left kidney was exposed, and the left renal artery was isolated and cleared of connective tissue and fat. A sterling silver renal artery clip (gap size, 0.2 mm) was slipped over the left renal artery close to the aorta. Clips were not placed on the renal artery of rats that underwent sham operations. The gut was then restored to the abdominal cavity, and both body wall and skin incisions were closed in layers with silk suture.

Blood pressures were measured routinely in all experiments by tail plethysmography in conscious rats using either the Doppler (Narco Bio-Systems, Houston, TX, USA) or photoelectric (IITC, Landing, NJ, USA) methods. With either method, rats were warmed for 20 minutes at approximately 37°C before recordings were made. At least five determinations of systolic pressure were made for each rat during each recording session, and the mean of the lowest three pressures within 5 mm Hg was used to calculate the systolic blood pressure value.

The procedure for determining aortic DNA synthesis was adapted from that of Khairallah et al.\(^18\) The rats were killed by decapitation, and the aortas from the heart to the femoral bifurcation were excised rapidly and placed in ice-cold normal saline. The loose connective tissue was removed with forceps, and the aorta was rinsed in fresh saline to remove blood and debris. The vessel segment was then cut lengthwise to expose the intima of the vessel and rinsed with ice-cold saline to remove any residual blood. The aortas were then blotted dry on filter paper, weighed, and placed into individual 25-ml Erlenmeyer flasks with airtight caps containing 20 ml of ice-cold Krebs-Henseleit buffer solution saturated with 95% oxygen, 5% carbon dioxide. The maximum time vessels were held in ice-cold solution was 40 minutes. The cleaning and weighing process appeared to remove the endothelial layer, since sections of sample vessels showed little, if any, remaining endothelium at the end of the procedure.

The flasks containing the vessel segments were preincubated for 60 minutes at 37°C in a shaking water bath. At the end of this preincubation period, \[^{3}H\]thymidine (1.0 mCi/ml; specific activity, 50 Ci/mmol; Moravek Biochemicals, La Brea, CA, USA) was added to the flask to achieve a total activity of 2 or 10 μCi/ml, as indicated. The flasks were incubated for 60 minutes. At the end of the incubation period, the tissues were rinsed three times with ice-cold saline. The tissue was then homogenized in 2 ml of 0.2 N perchloric acid (PCA) using a glass-on-glass hand-held homogenizer (Model 21; Kontes, Vineland, NJ, USA). The homogenate was used for DNA and protein determination.

Following homogenization, the samples were centrifuged for 10 minutes at 1000 g at room temperature. A 1.0-ml aliquot of supernatant was removed to determine acid-soluble radioactivity. In aortas treated with 2 μCi/ml labeled thymidine, this aliquot was found to contain 1110 ± 353 and 1060 ± 208 dpm/mg aorta in control rats and rats clipped for 5 days, respectively. In aortas treated with 10 μCi/ml labeled thymidine, this aliquot was found to contain 4473 ± 766 and 3565 ± 784 dpm/mg aorta in control rats and rats clipped for 5 days, respectively. This fraction represents the sum of thymidine and metabolites of thymidine that were not incorporated into DNA. These results show that the uptake of labeled thymidine was not different in aortas from control and clipped rats. The pellet was then washed with 0.2 N PCA and recentrifuged twice to remove any residual acid-soluble radioactivity from the pellet.

The pellet was then resuspended in 2 ml of 0.5 N
PCA and heated at 90°C for 20 minutes to hydrolyze and extract the DNA. After cooling, the samples were recentrifuged at 1,000 g for 10 minutes. The supernatant was used for DNA determinations, and the pellet was used to estimate protein. The pellet from each sample was resuspended in 2.0 ml of 1.0 N NaOH and incubated at 45°C for 1 hour to solubilize the proteins that were assayed using the Coomassie blue method.19

The incorporation of [3H]thymidine into DNA was quantified by pipetting 500 μl of supernatant containing the DNA hydrolysate into counting vials containing 10 ml of scintillation fluid (Liquiscint, National Diagnostics, Parsippany, NJ, USA). Aliquots were also used for the assay of DNA according to the method of Burton.20 Incorporation of [3H]thymidine into DNA was found to be linear for up to 2 hours. Since DNA synthesis was measured as [3H]thymidine incorporated into DNA per hour, the process is referred to in the text as the rate of DNA synthesis (expressed as dpm/μg of DNA).

In some experiments, [3H]thymidine incorporation into bone marrow DNA was determined as a measure of DNA synthesis in a rapidly proliferating tissue. To measure bone marrow DNA synthesis, the left femur was removed and cleaned of flesh and connective tissue. The semilunar cartilage was removed, revealing the bone marrow, and the head of the femur was severed to allow the marrow to flow out of the bone when flushed. A 22-gauge needle was inserted into the marrow, and the cavity was flushed with 4 ml of ice-cold, oxygenated Krebs-Henseleit buffer into a plastic centrifuge tube. This solution was rapidly diluted up to 20 ml with additional buffer. The bone marrow samples were then preincubated at 37°C for 60 minutes. After preincubation, 40 μCi of [3H]thymidine was added and the tissue was incubated for an additional 60 minutes. At the end of the incubation period, the tubes were centrifuged at low speed and the supernatant was removed. Then, 2 ml of 0.2 N PCA was added to each tube to lyse the cells. Incorporation of labeled thymidine into DNA was determined as described for the aorta.

To determine whether the rise in blood pressure in renal hypertension was dependent on DNA synthesis, the DNA synthesis inhibitor Ara-C (donated by Dr. R. Engle, National Cancer Institute or obtained from Sigma Chemical, St. Louis, MO, USA) was used to block DNA synthesis during the developmental phase of renal hypertension. Three groups of rats were used. Intraperitoneal Ara-C administration (100 mg/kg twice daily, dissolved in saline) was begun in one group on the day of clipping and continued for an additional 4 days. A second clipped group was given saline injections. Body weight, food and water intake, and urine output were measured daily. A third group of normal rats also was given Ara-C.

Histological techniques were used to confirm which cell types were being examined in the biochemical studies. The tissues used were obtained similarly to those used for the analysis of DNA synthesis. Following incubation with [3H]thymidine, the aortas from sham-operated rats and rats with renal hypertension of 5 and 21 days' duration were rinsed three times and fixed in a solution consisting of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.08 M cacodylate buffer, immersed in 2% osmium tetroxide for 3 hours, dehydrated in graded concentrations of ethanol, and then embedded in epoxy resin; 1-μm sections were cut and affixed to slides. These were stained and examined by light microscopy.

Data are reported as group mean ± 1 SEM. Significance of differences between treated and control groups was estimated using the Student's t test. Analysis of variance followed by the Newman-Keuls test was used to compare differences between more than two groups. A p value less than 0.05 was accepted as being statistically significant.

Results

After application of a 0.2-mm clip to the left renal artery, hypertension developed over a 2-week period. Table 1 shows that 5 days after clipping, and before the hypertension was manifest, there was a significant increase in the rate of aortic DNA synthesis, as measured by [3H]thymidine incorporation into aortic DNA. At this time, there was no correlation between blood pressure and the rate of [3H]thymidine incorporation into DNA (r = 0.323).

When tracer amounts of [3H]thymidine are used to estimate DNA synthesis, the specific activity of the endogenous thymidine that is incorporated into DNA is determined by the pool size of unlabeled thymidine in the cells in S phase. It is thus possible that the apparent six-fold increase in DNA synthesis observed in aortas from clipped animals was due to a decrease in the pool size of thymidine in the aortas of these rats to one sixth that of controls. This possibility is highly unlikely since there were no differences between groups in the uptake of labeled thymidine into the first supernatant after homogenization (see Methods). To further exclude this possibility, we measured the incorporation of [3H]thymidine using a five-fold higher concentration of labeled thymidine plus unlabeled thymi-

| Table 1. Blood Pressure and DNA Synthesis 5 Days After Renal Artery Clipping or Sham Operation |
|---------------------------------------------------------------|-----------------|-----------------|
| Parameter                                                      | Sham-operated   | Clipped         |
|                                                               | (n = 4)         | (n = 7)         |
| Systolic blood pressure (mm Hg)                                | 122 ± 3         | 123 ± 7         |
| DNA synthesis (dpm/μg DNA)                                     | 18.2 ± 4.4      | 111.6 ± 28.7*   |
| DNA (μg)/protein (mg) ratio                                   | 39.7 ± 1.5      | 41.1 ± 1.9      |
| DNA (μg/mg wet weight)                                        | 1.27 ± 0.05     | 1.20 ± 0.06     |
| Protein (μg/mg wet weight)                                    | 30.9 ± 1.7      | 30.6 ± 2.1      |

*p < 0.05, compared with values in sham-operated rats (by unpaired Student's t test). Values are means ± SEM. Renal hypertension was induced by placement of a 0.2-mm silver clip on the left renal artery. As described in Materials and Methods, DNA synthesis was determined 5 days after clipping or sham operation.
DNA to make a 10-fold higher concentration of total thymidine in the incubation mixture. The presence of the carrier thymidine would be expected to perturb the endogenous pools such that the specific activity of the thymidine incorporated into DNA could approach the specific activity of the added thymidine and therefore minimize any difference in endogenous pools. This effect probably was accomplished since there were no differences between sham-operated and clipped groups in uptake of labeled thymidine into the aortic homogenate in these experiments, as reported in Methods. As shown in Figure 1, the presence of carrier thymidine increased incorporation of the labeled thymidine into DNA by a similar amount in aortas from both control and clipped rats. Furthermore, under both incubation conditions, incorporation of [3H]thymidine was significantly greater in aortas from clipped rats. This experiment shows that the increased incorporation of [3H]thymidine into aortic DNA was not an artifact of altered pool sizes but represented increased DNA synthesis.

Aortic DNA synthesis was also determined 3 weeks after clipping, when blood pressure had become established. Table 2 indicates that there was a significant increase in [3H]thymidine incorporation into aortic DNA in the hypertensive animals when compared with that in the sham-operated controls. In addition, the significant decrease in the DNA/protein ratio suggests that cellular hypertrophy had occurred by 3 weeks.

Inhibition of DNA synthesis with twice daily injections of Ara-C (100 mg/kg) delayed the onset of renal hypertension, as shown in Figure 2. The Ara-C treatment per se did not have any direct effect on blood pressure, since blood pressure in the Ara-C-treated, normal rats was unaltered throughout the experiment. After clipping, there were no significant differences in blood pressure among the Ara-C–treated and saline-treated groups during the 5-day treatment period. Although blood pressure was not measured on Day 5 in this experiment, other experiments have consistently shown that the blood pressures in clipped and control animals do not differ at this time (see Table 1). There were also no differences in blood pressures between the two Ara-C–treated groups on Days 7 or 8. Blood pressure in the clipped, Ara-C–treated group did not begin to exceed preclipping levels until Day 9, which was 4 days after cessation of drug treatment, whereas in the non-drug-treated, clipped rats, systolic pressure had increased sharply by Day 7. The blood pressure of the clipped rats treated with Ara-C remained significantly lower than that of the saline-treated group until Day 11, which was 6 days after Ara-C had been discontinued. As shown in Figure 3, Ara-C–treated, clipped rats did not exhibit any significant alterations in body weight, food or water intake, or urine output.

Table 2. DNA Synthesis in Rat Aorta 3 Weeks After Clipping or Sham Operation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham-operated (n = 9)</th>
<th>Clipped (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>125 ± 2</td>
<td>205 ± 5*</td>
</tr>
<tr>
<td>DNA synthesis (dpm/μg DNA)</td>
<td>27.3 ± 5.0</td>
<td>165.0 ± 41.0*</td>
</tr>
<tr>
<td>DNA (μg)/protein (mg) ratio</td>
<td>43.2 ± 1.8</td>
<td>35.1 ± 2.2*</td>
</tr>
<tr>
<td>DNA (μg/mg wet weight)</td>
<td>1.45 ± 0.11</td>
<td>1.43 ± 0.15</td>
</tr>
<tr>
<td>Protein (μg/mg wet weight)</td>
<td>34.3 ± 3.5</td>
<td>42.5 ± 6.5</td>
</tr>
</tbody>
</table>

*p < 0.05, compared with values in sham-operated group (by unpaired Student’s t test).

Values are means ± SEM. The DNA synthesis was determined in hypertensive and sham-operated rats 3 weeks after operation, as described in Materials and Methods.

Figure 1. Effect of increasing total thymidine concentration on DNA synthesis in vitro in aortas from clipped (n = 7) and sham-operated (n = 4) rats 5 days after operation. Aortas were divided in half and incubated in either tracer thymidine or tracer thymidine supplemented with cold thymidine. BP = blood pressure.
FIGURE 2. Effect of cytosine arabinoside (Ara-C) treatment on blood pressure in rats with developing renal hypertension or in normal rats. Saline or Ara-C (100 mg/kg) was administered twice daily intraperitoneally. Each group contained nine or 10 animals. Effects were compared in Ara-C-treated, normal rats (○); Ara-C-treated, clipped rats (△); and saline-treated, clipped rats (○). Asterisk indicates significant difference (p<0.05) from values in Ara-C-treated, clipped rats.

when compared with the saline-treated, clipped group. Similarly, body weight was not altered in the normal rats receiving Ara-C.

To verify that Ara-C was inhibiting DNA synthesis, aortic and bone marrow DNA synthesis rates were determined on Day 5 after renal artery clipping, 12 hours after the last dose of Ara-C, and on Day 10, which was 5 days after Ara-C treatment was stopped. We chose to examine DNA synthesis in the bone marrow because this tissue normally has a high proliferative rate as compared with that in the aorta, where the DNA synthesis rate is normally very low. As can be seen in Figure 4, Ara-C treatment for 5 days caused a marked decrease in both aortic and bone marrow DNA synthesis. By Day 10, which was 5 days after cessation of Ara-C treatment, both aortic and bone marrow DNA synthesis had returned to levels not significantly different from those in the non-drug-treated animals (data not shown).

Discussion

These studies point out that blood pressure alone may not be the main stimulus for the increase in aortic DNA synthesis seen in renal hypertension. Since aortic DNA synthesis was increased 5 days after a clip was placed on the renal artery and before an increase in pressure was detected, it appears most unlikely that pressure was the stimulus to the increased DNA synthesis. Aortic DNA synthesis was still increased in the clipped animals compared with the sham-operated controls 3 weeks after clipping, at a time when blood pressure had plateaued. In addition, inhibition of DNA synthesis for 5 days delayed the onset of hypertension, suggesting that there could be an obligatory DNA synthetic step before the blood pressure increase.

Light microscopy revealed no gross vascular damage caused by the hypertension. Endothelial cell replication could not have contributed to the measurement of DNA synthesis since the endothelial cells were removed before exposure to the thymidine label. Although we examined aortic sections that had been processed for autoradiography, too few labeled cells
Asterisk indicates significant difference (p < 0.05) from values and Methods. There were at least three rats in each group.

Carlier et al. also showed increased levels of aortic DNA synthesis in the one-kidney, one clip model of renal hypertension. DNA synthesis was measured on the fifth day, 12 hours after the last Ara-C dose, as described in Materials and Methods. There were at least three rats in each group. Asterisk indicates significant difference (p < 0.05) from values in saline-treated, clipped group (p < 0.05).

The role of DNA synthesis was examined during the development of renal hypertension by determining whether the prevention of DNA synthesis could prevent the development of hypertension. Cytosine arabinoside is thought to inhibit proliferation by blocking DNA synthesis. The major mechanisms of this drug action are 1) inhibition of DNA polymerase II and 2) incorporation into DNA, resulting in inhibition of further chain elongation. 29

As shown in Figure 2, Ara-C treatment had no effect on blood pressure in the saline-treated or clipped rats on Day 4. Since blood pressure was not measured on Day 5 in these experiments, we cannot exclude the possibility that blood pressure may have increased by Day 5 in the data presented in Figure 2, even though other experiments have shown that the blood pressure in clipped and control animals does not differ at this time (see Table 1). Treatment with Ara-C did, however, inhibit both aortic and bone marrow DNA synthesis, as shown in Figure 4. In addition, Ara-C delayed the increase in blood pressure seen in the saline-treated, clipped rats. Blood pressure in the Ara-C-treated group of clipped rats was still significantly lower than that in the untreated, clipped rats up to at least 4 days after cessation of treatment. Although there is the possibility that increased DNA synthesis and increased pressure might be concurrent phenomena, these data suggest that DNA synthesis may be a necessary component in the chain of events that occurs after renal artery constriction and that ultimately produces sustained increases in blood pressure, since inhibition of DNA synthesis delayed the onset of the blood pressure increase.
Because Ara-C, a drug commonly used as an anti-neoplastic agent, can be extremely toxic, the delay in the onset of hypertension might be explained by drug toxicity. This possibility seems unlikely, however, since the clipped rats receiving Ara-C did not exhibit any alterations in body weight, food or water intake, or urine output during the course of the study when compared with clipped rats not receiving the drug (see Figure 3). In addition, neither blood pressure (see Figure 2) nor body weight was altered in normal rats receiving Ara-C. The dose of drug used (100 mg/kg b.i.d.) was much lower than the rat median lethal dose (>1000 mg/kg/day) or the 1000 mg/kg dose reported to cause leukopenia in rats.30 The dose of Ara-C used has been shown to be very effective in preventing glial proliferation in response to a cortical injury and the inhibition of normal proliferation of bone marrow in the rat.31

Treatment with Ara-C probably had little or no effect on the production of renin or angiotensinogen since it has very little effect on protein or RNA synthesis. Recent studies29,32 suggest that Ara-C is incorporated almost exclusively into DNA rather than RNA. Silagi33 has shown that RNA and protein synthesis were not affected in L cells exposed to lethal doses of Ara-C, and Ben-Porat et al.34 have demonstrated that Ara-C had no effect on the synthesis of pseudorabies virus structural proteins, or their movement from the cytoplasm to the nucleus, while it prevented the synthesis of viral DNA. Additionally, Tourtellotte et al.35 have shown that intravascular administration of Ara-C had no effect on de novo central nervous system immunoglobulin G synthesis in humans in spite of severe granulocytopenia and monocytopenia of 1 or 2 weeks' duration. In patients given the drug intrathecally, cytoxic levels were maintained in the central nervous system for over 24 hours with no evidence of altered immunoglobulin synthesis or leukopenia. Thus, the effects we saw in our experiments were most likely due to the antiproliferative actions of the drug, although we cannot exclude the possibility that Ara-C could affect the synthesis of, or the response of the animals to, humoral factors that could affect blood pressure.

Thus, DNA synthesis may be involved in the development of renal hypertension. Five days after renal artery clipping and before hypertension was manifest, there was an increase in aortic DNA synthesis. This increase was also present 3 weeks after clipping, when the blood pressure had plateaued at a higher level. Inhibition of DNA synthesis with Ara-C was shown to delay the blood pressure increase, suggesting that the stimulus for the increase in blood pressure might be some factor whose synthesis occurred subsequent to synthesis of DNA. These findings suggest that an increase in DNA synthesis may be an obligatory step in the genesis of renal hypertension.

Acknowledgment
The authors thank Mr. Willie Wynne for his expert technical help.


Increased aortic DNA synthesis precedes renal hypertension in rats. An obligatory step?
A L Loeb, H G Mandel, J A Straw and B L Bean

Hypertension. 1986;8:754-761
doi: 10.1161/01.HYP.8.9.754
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1986 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/8/9/754