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Increase by adrenaline or angiotensin II of the accumulation of low density lipoprotein and fibrinogen by aortic walls in unrestrained conscious rats

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1 Earlier experiments of ours with anaesthetized rabbits showed that infusions of catecholamines into the carotoid blood stream significantly increased the uptake of radioiodinated low density lipoprotein (LDL) by the artery wall after as little as 2 h. This observation has now been extended to much longer time periods, i.e., 6 days, and another species, viz. conscious and unrestrained rats; also to another pressor agent, angiotensin II, as well as to another plasma protein, fibrinogen.

2 Groups of rats were infused from subcutaneously implanted osmotic minipumps for 6 days. The infusions were either into a carotid artery or into the surrounding tissues, with essentially the same effects. Control animals were infused with saline, and test animals with either adrenaline or angiotensin II. The minipump concentration of adrenaline of 4–5 μM, which gave blood concentrations of 25–41 nM, increased the blood pressure significantly after 3 days. The minipump concentration of angiotensin II of 9.9 mg ml^{-1} was chosen to produce similar increases in blood pressure.

3 Five days after starting the infusion, rats were injected i.v. with either homologous or human LDL labelled with [^{125}I]-tyramine cellobiose (TC), or with [^{131}I]-TC labelled human fibrinogen. Twenty-four hours later, the animals were killed and the radioactivities determined in the whole aorta. The labelled TC radioactivities represent primarily metabolised protein (because TC is trapped intracellularly), but also include the fraction of intact, i.e., non-metabolized protein in transit through the vessel wall. To determine the contribution of the latter, in some experiments we injected double-labelled [^{131}I]-[^{125}TC]-LDL only. These experiments showed that the [^{131}I]-LDL counts representing protein in transit accounted for approx. 20% of the total ^{125}TC counts, and that this percentage was not significantly affected by adrenaline or angiotensin II. Therefore, the bulk of the experiments was carried out with single labelled proteins, using ^{125}I to label TC-LDL and ^{131}I to label TC-fibrinogen. In these experiments, the radioactivity of the arterial wall thus provides a cumulative measure of the uptake and degradation of proteins.

4 Aortic wall radioactivities from rat and human LDL and from human fibrinogen were significantly increased by both agents. Adrenaline at 25–41 nM increased the radioactivities by 52 and 47% for rat and human LDL respectively, and by 31% for human fibrinogen; these differences were highly significant ($P<0.01$). Angiotensin II at ca. 10 nM also increased the radioactivities significantly, by 21% for human LDL and by 109% for human fibrinogen ($P<0.05$).

5 The results suggest that the accumulation of LDL and of fibrinogen by rat aorta is increased by adrenaline or by angiotensin II at concentrations which raise the blood pressure progressively and significantly after 3 or 5 days respectively.

Keywords: Adrenaline; blood pressure; low density lipoproteins (LDL); fibrinogen; uptake; conscious rat

Introduction

The first indication of atherosclerosis is the accumulation of lipid in the intima of susceptible arteries. These fatty streaks, classified by the World Health Organisation as Stage I of atherosclerosis, are characterized by extracellular and intracellular lipids and are usually covered by intact endothelium. The lipid is predominantly derived from low density lipoprotein (LDL) of the plasma. The lesions also contain fibrinogen and fibrin in considerable amounts (Smith & Crosbie, 1992).

The only established rate-determining factor of the arterial accumulation of LDL is its concentration in the plasma (Goldstein & Brown, 1977; Nagelkerke *et al.*, 1984; Brown & Goldstein, 1986). In looking for other possible rate determinants we thought of hypertension as an established risk factor for coronary heart disease as well as for stroke (Collins *et al.*, 1990; MRC Working Party, 1992). We provided evidence that in anaesthetized rabbits, the amount of LDL in artery walls is increased by low concentrations of the pressor amines noradrenaline and adrenaline (Shafi *et al.*, 1989; Cardona-Sanclemente & Born, 1991); and a similar effect was observed in conscious, unrestrained rats (Cardona-Sanclemente *et al.*, 1992). The experiments showed that the catecholamines increased artery wall radioactivities derived from intravenously injected LDL which had been radio-iodinated via the adduct tyramine cellobiose (TC). The radioactive adduct remains in the tissues when LDL is degraded. In previous papers we suggested that the arterial wall radioactivities 24 h after LDL injection were predominantly a measure of uptake, although we were aware that there would be a contribution from undegraded LDL in passage through the walls. Having in this paper determined the contribution of undegraded LDL and found it to be minor, i.e. ca. 20%, we now refer to the radioactivities as measures of LDL accumulation.

The flux of plasma macromolecules into artery walls has

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been much investigated experimentally, mostly with albumin, *in vitro*, and over short time periods (see, for example, Baldwin *et al.*, 1983). Albumin was also used in some *in vivo* experiments, two of which reported effects of blood pressure (Duncan & Cornfield, 1982; Feig *et al.*, 1982). The latter provided evidence that in anaesthetized rabbits, brief, i.e. 30 min, infusion of angiotensin II at concentrations which caused great, i.e. 60 mmHg, but transient rises in blood pressure increases the uptake of radio-iodinated albumin in the arch but not in the descending part of the aorta.

Amongst the questions arising from our earlier observations were, whether similar effects might be demonstrable for other atherogenic plasma proteins and also with other pressor agents. In this paper we show with validated methods that in conscious, unrestrained rats the arterial accumulation of LDL and of fibrinogen is increased by angiotensin II as well as by adrenaline, in association with moderate rises in blood pressure.

Methods

Animals

The experiments were done with male Wistar rats weighing 300–350 g which were fed a standard diet *ad libitum*. The rats were kept in an air-conditioned animal house with a light period lasting from 08 h 00 min to 20 h 00 min.

Low density lipoprotein preparation

Low density lipoprotein was isolated from human blood obtained by venepuncture from healthy students or laboratory personnel with lipid levels in the normal range. After mixing with 0.15 M EDTA, the blood was centrifuged at 800 *g* for 30 min at 4°C. Low density lipoprotein (LDL, density 1.019–1.063 g ml^{-1}) was separated by sequential centrifugation at 105000 *g* for 24 h at 4°C on KBr solutions adjusted to a final density 1.019 and 1.063 (Havel *et al.*, 1955; Koga *et al.*, 1969; Fidge & Poulis, 1976). The final LDL fraction was dialysed exhaustively at 4°C against phosphate buffered saline containing 0.3 mM EDTA at pH 7.5 to remove excess salts. Protein content was measured by BCA assay reagent (Pierce Chemical C., U.S.A.).

Rats under anaesthesia (see below) were killed by intra-aortic punture. The blood of each rat was collected into 4% Na_2EDTA and centrifuged at 2200 *g* for 20 min at 4°C. Pooled plasma from four rats was used for lipoprotein isolation as previously described (Cardona-Sanclemente *et al.*, 1988).

Fibrinogen

Lyophilized human fibrinogen (KaviVitrum, Sweden) was dissolved in physiological saline to give a concentration of 100 mg ml^{-1}.

Labelling and iodination

LDL and fibrinogen were radioiodinated by an adduct of cellobiose and tyramine (TC) radiolabelled with ^{125}I or ^{131}I and attached covalently to LDL or fibrinogen respectively (Pittman *et al.*, 1983). TC is not metabolized and remains trapped intracellularly when the lipoprotein or fibrinogen to which it is attached undergoes degradation. Therefore the radioactivity of the labelled TC was a measure of the proteins that were metabolized intracellularly. The labelled proteins were dialysed exhaustively in cold phosphate-buffered saline pH 7.5 containing 2 mM EDTA, and sterilized by filtration. The tyramine cellobiose-labelled LDL ([^{125}I]-TC-LDL) or fibrinogen ([^{131}I]-TC-fibrinogen) had specific activities of 100–400 c.p.m. ng^{-1} protein and a free ^{125}I content of 1–4% for LDL, and of 50–150 c.p.m. ng^{-1} protein and a free ^{131}I content of 1–3.5% for fibrinogen.

The TC-associated radioactivities provided a measure of two fractions of proteins. One fraction represents the protein metabolized in the aortic walls over 24 h. The other fraction consists of intact labelled protein currently in transit through the wall. To measure the proportion of intact protein, human LDL was doubly labelled in some experiments. After labelling with ^{131}I the protein was passed through a Sephadex G-10 column. Then the [^{131}I]-LDL fraction was labelled with [^{125}I]-TC, and dialysed exhaustively. The specific activities and free iodine of the injected material were as above (Pittman *et al.*, 1983).

To find out whether free iodine radioactivity would significantly affect the total radioactivity measured in the aortic walls after 24 h, an experiment was done in which the ^{125}I and ^{131}I radioactivities were compared in two halves of longitudinally divided aortae. One half was treated and counted as usual; the other half was homogenized, precipitated with 10% trichloroacetic acid (TCA), and the radioactivities of the pellets were determined.

For the main series of experiments the solutions of [^{125}I]-TC-LDL and [^{131}I]-TC-fibrinogen were mixed together, diluted with sterile saline so that each rat received no more than 7% of its blood volume, injected into the femoral vein and allowed to circulate for 24 h. The injected volumes were standardized with respect to the rats' body weights in order to ensure that the initial blood radioactivities were the same in each rat.

Administration of compounds

Adrenaline or angiotensin II were infused by implanted osmotic minipumps (model 1007D; Alza Corp., Palo Alto, U.S.A.). The pumps contained adrenaline at a concentration of 9.6 mg ml^{-1}, i.e. ca. 4 μM, or 48 mg ml^{-1}, i.e. ca. 20 μM, together with ascorbic acid 10 mg ml^{-1}; angiotensin II at a concentration of 9.0 mg ml^{-1}, i.e. ca. 9 μM; or saline in control rats. The pumps delivered the solutions at a rate of 0.5 μl h^{-1} over 6 days.

Surgical procedure

Rats were anaesthetized with intramuscular fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg ml^{-1} (Hypnorm Janssen) 0.3 ml kg^{-1} and intraperitoneal methyl diazepin-one (Diazepam, Phoenix Pharm) 2.5 mg kg^{-1}. The operative procedure was as follows: in groups of rats, pumps containing adrenaline, angiotensin II or saline were implanted under the skin of the neck. For delivering drugs into the circulating blood, the implanted minipump was connected via a polythene catheter (0.80 mm external diameter) to the right common carotid artery. For delivering drugs directly into the subcutaneous tissues, the minipump was implanted under the skin. Five days after implanting the minipumps the rats were anaesthetized with Hypnorm only and labelled proteins were injected through the femoral vein. In groups of four rats, 25 μl samples of tail-vein blood were taken and the radioactivities determined to measure elimination of the labelled proteins from the circulation. Twenty four hours later the rats were killed by intra-aortic puncture under Hypnorm and Diazepam. The whole length of the aorta was exposed from the heart to the bifurcation and excised intact. Fat and loose connective tissue surrounding the adventitia were removed under a stereomicroscope and the vessel was carefully cleaned and opened longitudinally. The vessel was washed 5 times for 15 min in 50 ml ice-cold saline, followed by a washing in 5% KI in 0.9% saline, and placed in preweighed plastic tubes. After radioactivity counting the tubes were dried to constant weight at 60°C for at least three days. LDL protein (apoB) was determined by standard methods (Lowry *et al.*, 1951). Results are expressed as ng of protein mg^{-1} dry weight. Body weight was determined before and 120 h after minipump implantation and at the end of the experiment. All procedures were approved by the Animal Care and Safety Committee of the William Harvey Research Institute and conformed to guidelines established by the Home Office.

Adrenaline quantification

For determining adrenaline concentrations, samples were collected from larger minipumps (model 2ML4; delivery rate 2.5 $\mu l\ h^{-1}$ over 28 days) which still contained infusate after 6 days. The adrenaline concentration was adjusted to ensure infusion of the same concentration of adrenaline as when 1007D minipumps were used. Adrenaline was also determined in the blood of groups of rats at 2-day intervals during the infusion period. The blood was immediately mixed with heparin and EDTA and centrifuged at 2500 *g* for 5 min at 2°C to remove the cells; the plasma was stored at −70°C. Adrenaline was quantified by double-isotope radioenzymic assay (Brown & Jenner, 1981).

Blood pressure measurements

The rats' blood pressure and heart rate were determined from the carotid artery after 2, 24, 48 and 120 h. The measurements were made on groups of conscious unrestrained rats receiving saline, adrenaline or angiotensin II from implanted minipumps, by connecting an indwelling catheter in the carotid artery to a transducer coupled to a Phillips blood pressure recorder, which measured heart rate at the same time.

Statistical analysis

The significance of differences among groups of animals was assessed by the two-way ANOVA test used to compare means among different groups. The plasma clearance in each test group at each time point was tested by Mann Whitney U-test. In both statistical analyses a P value of less than 0.05 was considered significant. Results are showed as mean ± s.e.mean.

Results

The stability of adrenaline in the implanted minipumps and its concentration in the infused rats' blood are shown in Figure 1. In the minipumps the concentration from the second to the sixth day of infusion remained between 4 and 5 μM. In the blood the adrenaline concentration was about 12 nM before infusion, increasing to 34 after 2 days' and to 41 nM after 4 days' infusion, with a decrease to 25 nM after 6 days' infusion.

The effect of adrenaline infused by minipumps containing 4–5 μM (see Figure 1) on the rats' diastolic blood pressure is shown in Figure 2. In the control rats the blood pressure decreased moderately over 5 days. In rats infused with adrenaline the blood pressure increased progressively, with the difference from the controls significant after 3 and highly significant after 5 days. On this basis, a minipump concentration of angiotensin II (i.e. 9.8 mg ml^{-1}) was chosen which produced an increase in blood pressure similar to that of adrenaline (Figure 2). These adrenaline and angiotensin II concentrations had no significant effect on heart rate during the infusion period (Figure 3).

Plasma eliminations were determined in the same rats for both rat and human LDL by labelling them with ^{125}I and ^{131}I respectively (Figure 4). The times taken for plasma radioactivities to decrease to one-half of initial values are shown as $t_{½}$. The elimination of rat LDL ($t_{½}$ = 4.8 ± 0.3 h) was faster than that of human LDL but was not significantly affected by either adrenaline or angiotensin II. The elimination of human LDL ($t_{½}$ = 13.6 ± 1.1 h) was not affected by adrenaline and not significantly accelerated by angiotensin II ($t_{½}$ = 11.3 ± 1.2 h). The plasma elimination of human fibrinogen ($t_{½}$ = 3.9 ± 0.4 h) was not significantly affected by adrenaline or angiotensin II (Figure 5).

In rats in which adrenaline had been infused intraarterially for 5 days to produce blood concentrations of approx. 25–40 nM, the aortic wall radioactivity 24 h after i.v. injection of [^{125}I]-TC-rat LDL was increased significantly over controls (Table 1). When the infused adrenaline concentration was increased five fold, the percentage increase in accumulation was three times greater. Under the same experimental conditions adrenaline significantly increased the aortic accumulation of

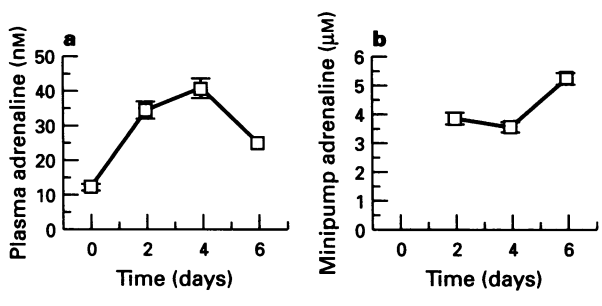

Figure 1 Adrenaline concentrations in plasma and in minipump infusate during six days of subcutaneous infusion into unrestrained conscious rats, n=5. The values represent means ± s.e.mean: (a) plasma concentration (nM); (b) minipump concentration (μM).

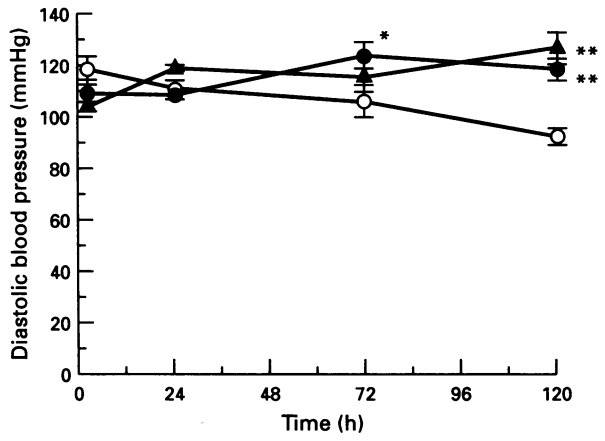

Figure 2 Comparison of the effect of saline (○) against that of adrenaline ca. 40 nM (●) or of angiotensin II ca. 10 nM (▲) infused subcutaneously by osmotic minipumps on the diastolic blood pressure of conscious, unrestrained rats, n=5. The values represent means ± s.e.mean between groups at the end of the 6 days infusion: $*P<0.05$, $**P<0.01$.

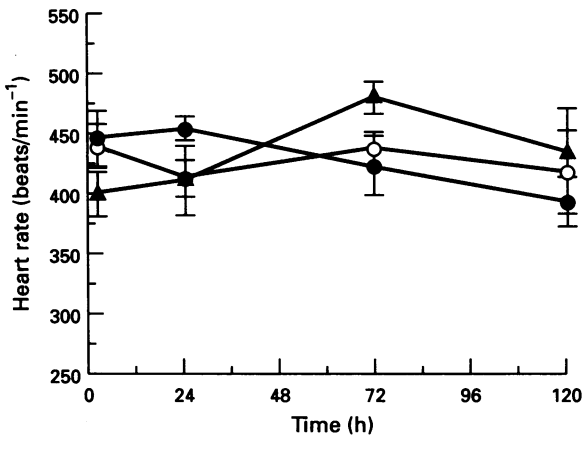

Figure 3 Effect of saline (○), adrenaline ca. 40 nM (●) or angiotensin II ca. 10 nM (▲) infused subcutaneously by osmotic minipumps on the heart rate of conscious, unrestrained rats, n=5. The values represent mean ± s.e.mean.

human LDL. In the same experiment, another group of rats was infused with angiotensin II instead of adrenaline. The angiotensin significantly increased the accumulation of human LDL. Again under the same experimental conditions, infusion of adrenaline or of angiotensin II also increased the accumulation of human fibrinogen by aortic walls.

The above results were validated by two further experiments, as follows: comparison of radioactivities from longitudinally divided halves of aortic walls, where one half was determined directly and the other after precipitation with TCA, showed that the latter were consistently lower by between 10 and 24%; however, these values were not significantly different from the controls (Table 2). Therefore, the amounts of free iodine and/or radioactive iodine attached to TCA-soluble fragments made no significant difference to the results.

The other validating experiment was to find out what proportion of the TC-radioactivities might be attributed to intact protein currently in transit through the aortic wall. For this experiment, the same preparation of human LDL was double-labelled with ^{131}I and then with [^{125}I]-TC. The results are shown in Table 3. Under the conditions of this experiment, the ratio of intact LDL to degraded LDL after 24 h was about 20%; this ratio did not differ significantly between control animals and those infused with adrenaline or with angiotensin II. These results show that the amount of LDL that had been degraded in aortic walls over 24 h greatly exceeded the amount of LDL in transit through the wall, and that this was not measurably changed by either adrenaline or angiotensin II as administered in these experiments. Thus, the measurements obtained with single-labelled proteins shown in Table 3 indicate the influence of catecholamines on the amount of protein that had penetrated the aortic wall and been metabolized by cells over a 24 h period.

Discussion

The results show that in conscious, unrestrained rats, adrenaline or angiotensin II infused by implanted minipump for six days significantly increased aortic wall radioactivities derived from radioiodine-labelled LDL or fibrinogen which had been circulating for 24 h. These radioactivities represent the radioiodinated tyramine cellobiose of degraded LDL and of undegraded LDL currently passing through the artery walls. We have now shown that the latter accounts for only about 20% of the total radioactivities, so that they overwhelmingly re-

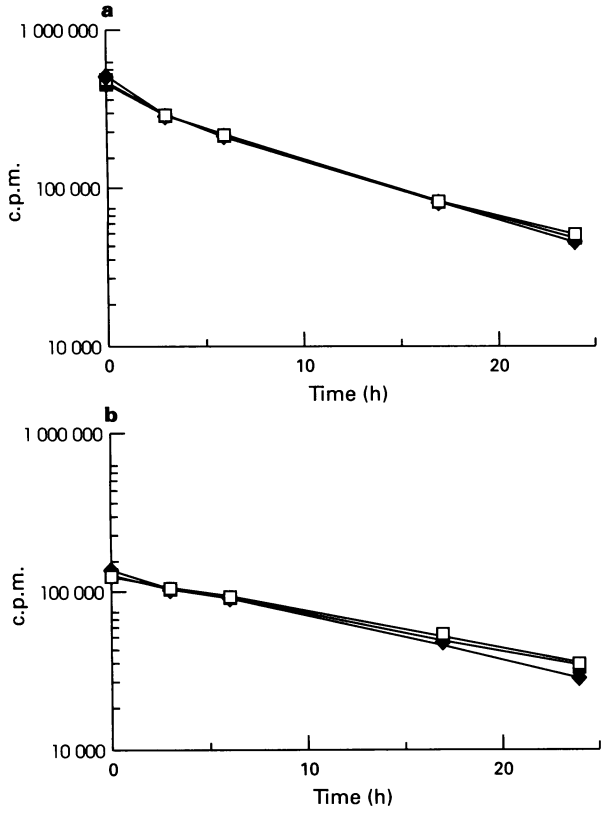

Figure 4 Plasma elimination of [^{125}I]-TC-ratLDL (a) and [^{131}I]-TC-humanLDL (b) in Wistar rats during the final 24 h of 6 days infusion with saline (i.e. control), adrenaline or angiotensin II. ($n=4$ per group), plotted semilogarithmically. Ordinates: % remaining; abscissae: time (h). (□) Control; (●) angiotensin II; (■) adrenaline.

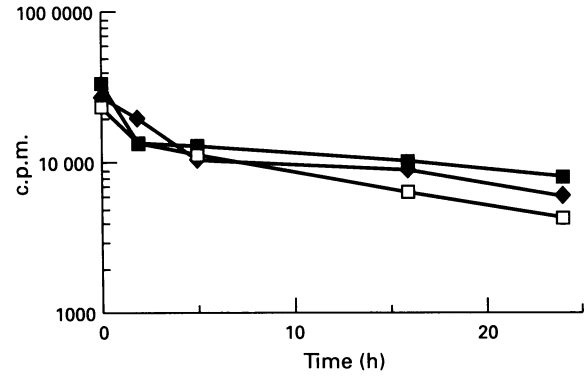

Figure 5 Plasma elimination of [^{131}I]-TC-human fibrinogen in Wistar rats ($n=4$ per group) during the final 24 h of 6 days infusion with saline (i.e. control), adrenaline or angiotensin II, plotted semilogarithmically. Ordinate % remaining; abscissae: time (h). (□) Control; (◆) angiotensin II; (■) adrenaline.

Table 1 Rat and human LDL and human fibrinogen in aortic walls of conscious unrestrained rats after six days' infusion from implanted minipumps of adrenaline or of angiotensin II

	Aortic radioactivities (control = 100%) of		
	Rat LDL $n=11$	*Human LDL* $n=14$	*Human fibrinogen* $n=12$
Blood conc. (nM)			
Adrenaline 25–41 (measured)	152**	147**	131**
Adrenaline ca.200 (estimated)	225*	–	–
Angiotensin II ca.10 (estimated)	–	121*	209*

For the rat LDL experiments the infusions were intra-carotid; for all other experiments, infusions were subcutaneous. The values represent means ± s.e.mean: *$P<0.05$; **$P<0.01$.

Table 2 Comparison of aortic wall radioactivities, with and without precipitation by trichloroacetic acid (TCA) (c.p.m. mg^{-1} dry weight)

		Fresh tissue ($n=5$)	*TCA-precipitated* ($n=5$)	*Decrease* (%)	P
Control	LDL	0.31 ± 0.02	0.28 ± 0.02	9.6	NS
	Fibrinogen	1.33 ± 0.17	1.17 ± 0.17	10.5	NS
Adrenaline	LDL	0.50 ± 0.02	0.43 ± 0.02	14.0	NS
	Fibrinogen	1.33 ± 0.17	1.00 ± 0.27	24.0	NS
Angiotensin	LDL	0.78 ± 0.10	0.62 ± 0.07	20.5	NS
	Fibrinogen	2.67 ± 0.58	2.17 ± 0.33	18.7	NS

Table 3 Ratio of [^{131}I]-LDL/[^{125}I]-TC-LDL in aortic walls (c.p.m. mg^{-1} dry weight, $n=5$)

	[^{131}I]-LDL	*[^{125}I]-TC-LDL*	*[^{131}I]-LDL* (%)
Control	0.07 ± 0.003	0.43 ± 0.02	16
Adrenaline	0.08 ± 0.003	0.48 ± 0.02	17
Angiotensin	0.13 ± 0.006	0.58 ± 0.02	22

present the accumulation of labelled tyramine cellobiose derived from LDL that was metabolized in the 24 h period. The effect has so far been demonstrated at this one time point because the tyramine-cellobiose label begins to leak out of tissues after longer periods (Pittman *et al.*, 1983).

Adrenaline or angiotensin II infused for 6 days by osmotic minipumps, whether subcutaneously or directly into the blood stream, increased the accumulation of rat and human LDL and of human fibrinogen.

Adrenaline or angiotensin II infused subcutaneously had no significant effect on the elimination of rat and human LDL or human fibrinogen from the plasma. Therefore, the increased accumulations caused by the agents could not be attributed to an effect on the concentrations of the labelled proteins in the circulating blood. Adrenaline increased the accumulation of both rat and human LDL in experiments in which the accumulation of human LDL in the control was considerably greater than that of rat LDL. This is consistent with the slower elimination of human than of rat LDL.

Angiotensin II was used to find out whether another pressor agent would act like adrenaline; and the results show that the accumulation of LDL and of fibrinogen is also increased by angiotensin II. At the concentrations infused, both adrenaline and angiotensin II increased the blood pressure moderately and progressively over 6 days. Compared to saline-infused control rats, in which the diastolic blood pressure gradually decreased, the increase with adrenaline became significant after 3 days and with angiotensin II after 5 days. The small continuous increase in the blood pressure in the control rats is commonly observed in such experiments and accounted for by their accomodation to the experimental situation, being kept caged in groups up to the surgical procedure, and afterwards in individual cages. The anaesthesia used for the surgical procedure may also have contributed to the initial decrease (Buelke-Sam *et al.*, 1978; Parker & Adams, 1978). As shown in Figure 2, at 2 and 24 h, the blood pressures in the other groups were not significantly different from the control group. Subsequent experiments, which have shown similar increases in accumulation accompanied by increases in blood pressure, have not shown this effect (Cardona-Sanclemente & Born, 1995). For that reason we decided that the first blood pressure measurement should be done at 24 h after cannulation.

The results presented in this paper would be compatible with the assumption that the increased accumulation of LDL and fibrinogen by aortic walls caused by adrenaline or by angiotensin II is mediated by a mechanism involving their pressor actions. This assumption would appear to be supported by our more recent findings that increases in blood pressure caused by inhibiting nitric oxide synthesis with L-NAME are also associated with increased accumulations of LDL and fibrinogen by aortic walls in such rats (Cardona-Sanclemente & Born, 1995). Indeed there is evidence that the entry of macromolecules into endothelial vesicles increases with intravascular pressure (Moffitt *et al.*, 1992). This conclusion would help to explain why hypertension is a risk factor for myocardial infarction and stroke.

However, other recent experiments (Cardona-Sanclemente *et al.*, 1994a, b) suggest that the aortic accumulation of LDL and fibrinogen can be increased by angiotensin II even when the pressor effect is small and transient. Furthermore, in spontaneously hypertensive Wistar rats of the same age as those used here, in which the diastolic blood pressure throughout the six-day experimental period was higher than that produced by the largest dose of angiotensin II, the accumulation of LDL and fibrinogen did not differ significantly from that in the control rats. Thus, the results indicate that the accumulation of LDL or of fibrinogen by rat aorta is increased by adrenaline or by angiotensin II, but provide no information as to the responsible mechanism(s).

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