Diabetes mellitus is associated with a notable increase in the incidence of cardiovascular disease. So-called diabetic macroangiopathy causes obstruction of large and medium-sized arteries, in which accelerated atherosclerosis takes a prominent (although not exclusive) part. The pathogenesis of macroangiopathy is not fully understood. A role could be played by the sustained increase in plasma insulin concentration, which is commonly present in diabetic patients, either spontaneously or as a result of treatment. Although still controversial, the link between chronic hyperinsulinemia and arterial disease is suggested by experimental and epidemiological observations and could extend beyond diabetic macroangiopathy to involve the increased risk of atherosclerosis associated with situations of insulin resistance such as obesity.

An important issue of the "insulin hypothesis" is to assess whether insulin directly modifies the metabolism of arterial smooth muscle cells that play a prominent role in atherogenesis. Many experimental studies done in vivo and in vitro (e.g., isolated artery or cell cultures) have examined the metabolic effects of insulin on smooth muscle. As reviewed in detail previously, the results have been contradictory. In vivo, there is a correlation between insulinemia and the metabolic activity of the arterial wall. In vitro, insulin enhances the synthetic activities of cultured arterial smooth muscle cells; however, there is no substantial increase in the metabolism of the intima-media when an isolated artery is incubated in the presence of insulin, even at a very high concentration. To explain this paradox, we have found that the glucose metabolism of intima-media from an isolated perfused artery is stimulated when exposure to insulin is performed under high hydrostatic pressure. Yet, this "hemodynamic" explanation is weakened by the fact that a quite unphysiological concentration of insulin (10^5 munits/ml) is required to observe this effect.

In the present series of experiments, we have tested an alternative "phenotypic" explanation. Smooth muscle cells express at least two phenotypes: the contractile phenotype, which is mainly devoted to muscular activity, and the secretory or synthetic phenotype, which is characterized by an intense synthetic activity and the ability to prolifer-
ate. The discrepancy between in vitro studies could be accounted for if it could be demonstrated that secretory cells (the phenotype adopted by cells in culture) directly respond to insulin, but that, in contrast, contractile cells (the phenotype of muscle cells present in a normal arterial wall) do not respond to insulin. If such were the case, then the apparent insulin sensitivity of normal arteries observed in vivo would be of an indirect nature.

To test this hypothesis, we injured rat thoracic aortas with a balloon catheter to induce phenotypic modulation of smooth muscle cells (from the contractile to the secretory state), and we explored the influence of insulin on the lipid synthesis of glucose by intima-media under two conditions: incubation of the aortas or exposure of the perfused aortas to insulin under physiological pressure. In the perfusion experiment as a control, we also studied the metabolism of the adventitia, the external arterial layer that is highly sensitive to insulin because it is rich in fibroblasts and adipose cells.

**Methods**

**Animals**

We used male Wistar rats (Iffa Credo, l’Arbresle, France) weighing 300–350 g. Before the rats were killed and their aortas incubated, all animals were fasted for 24 hours to establish a baseline metabolic state because feeding greatly influences the glucose metabolism and insulin sensitivity of arterial tissue.

All aggressive procedures (aortic catheterization, preparation to aortic perfusion, killing) occurred with the rats under ether anesthesia. The rats were handled and treated in accordance with the guidelines for the care and use of animals approved by the Council of the American Physiological Society.

**Aortic Injury**

As previously described, a deflated balloon embolectomy catheter (Fogarty size 2F; American Edwards Laboratories, Santa Ana, Calif.) was introduced into the aorta via the left common carotid artery to the level of the renal arteries. The balloon was inflated with 50 μl distilled water with a 1-ml syringe, and the catheter was withdrawn slowly. More water was injected into the balloon after the passage of the diaphragm had been felt, when the caliber of the aorta becomes slightly larger. Water was injected until a strong resistance to pushing the plunger of the syringe was encountered, which amounted to an additional volume of 40–60 μl, and the catheter was pulled up to the origin of the left common carotid artery. The balloon was passed in the aorta under the same conditions three times. After removal of the catheter, the left carotid artery was doubly ligated, and the incision was closed with surgical staples. Control rats with noninjured aortas were sham operated: the same procedure was done except that no catheter was introduced in the carotid artery.

Under such conditions of “hard injury,” complete endothelial denudation strongly stimulates DNA synthesis by the intima-media; the reaction is at its peak (at least a 30-fold increase compared with the control noninjured aorta) 2 days after injury and declines progressively thereafter, while smooth muscle cells migrate and divide in the intima. Fourteen days after injury, DNA synthesis has almost declined to that of the control level, but the intima has been thickened by an accumulation of smooth muscle cells.

**Incubation and Metabolic Study of Aortas**

Immediately after death or at the end of in situ perfusion (see below), the segment of each aorta between the left subclavian and celiac arteries was removed, opened longitudinally, and transferred into a 10-ml Erlenmeyer flask containing 2 ml Krebs-Henseleit bicarbonate (KHB) buffer with glucose (5 mmol/l), bovine serum albumin (10 g/l), soybean trypsin inhibitor (10 μg/ml), and ethylenediaminetetraacetic acid (EDTA) (10 mmol/l). The flask was gassed with 95% O2/5% CO2 (vol/vol). The medium was equilibrated at 37°C and pH 7.4 by gassing with 95% O2/5% CO2 (vol/vol). The flask was stopped with a serum cap, placed into a shaking metabolic incubator set at 37°C and 70 linear oscillations/min, and flushed for 2 minutes with O2/CO2. The incubation time was 1 hour. Metabolic activity of the tissue was stopped by injecting 0.1 ml 4N H2SO4 into the flask. The aorta was rinsed with KHB buffer and dissected into intima-media and adventitia layers. Lipids were extracted, and the amount of glucose equivalent accumulated as 14C lipids in the tissue, along with its DNA content, was measured as previously described. We took lipid synthesis from glucose as the sole metabolic index because we have previously found it to be the most sensitive criterion of the insulin sensitivity of arteries (as compared with CO2 and lactate production from glucose).

**Experimental Design and Exposure to Insulin**

**Experiment 1.** This was a broad screening to determine whether any obvious insulin sensitivity of the intima-media appeared during the 14 days after aortic injury. We studied five groups of eight rats: One group consisted of control sham-operated animals (D0), and the other four groups consisted of rats killed 1 (D1), 2 (D2), 8 (D8), and 14 (D14) days after aortic injury. Each aorta was incubated immediately after death in the absence (control [C], n=4) or presence (Ins; 1 unit=40 μg=7 nmol insulin; n=4) of insulin at a very high concentration (105 units/ml). Only the metabolism of the intima-media was studied. Because one rat died as a result of aortic catheterization, there were three instead of four rats in the C group on D14.

**Experiment 2.** Considering the negative results of experiment 1 (see below), we decided to examine more closely the effects of insulin on D2. To take the possible influence of hydrostatic forces into account, the aorta was exposed to insulin during a 9-minute...
perfusion under physiological pressure. The technique of in situ aortic perfusion was similar to that described previously except that: 1) The shunting line (used to avoid any collapse of the aorta during preparation) was inserted in the right common carotid artery (because the left one had been ligated during sham or real aortic catheterization). 2) The perfusion line was inserted into the left ventricle (instead of the common carotid artery) to use a larger cannula, allowing a higher flow rate (about 30 ml/min). The perfusion medium was the same as the incubation buffer, except that [14C]glucose was omitted. After perfusion, the aorta was incubated for 1 hour, as described above, with no insulin added to the medium. D0 aortas (n=8) perfused without insulin served as controls. D2 aortas were perfused either with no insulin or with 10^{-2} (n=8), 10^{-3} (n=7), 10^{-4} (n=8), or 10^{-5} (n=4) units/ml insulin added to the perfusion fluid. In this experiment, lipid syntheses from glucose by the intima-media and by the adventitia were compared.

Units and Statistics

Metabolic activity is expressed as picomoles of glucose-equivalent transformed in lipids per microgram DNA per minute of incubation. The amount of glucose converted to lipids was calculated by dividing the disintegrations in the product per minute by the specific activity of the glucose in the medium.

We used the Mann-Whitney U test to compare small groups (C and Ins in experiment 1)\textsuperscript{14}; these results are summarized by the median and the range. In all other cases, the results are summarized by the mean and 95% confidence intervals (95% CI) and have been compared with parametric tests: one-way analysis of variance (ANOVA) for comparisons between more than two groups, followed by Bonferroni's modified t test for further preplanned pairwise comparisons\textsuperscript{8}; and linear correlations for grouped samples.\textsuperscript{16} The values of lipogenesis by the adventitia (experiment 2) were analyzed after a logarithmic transformation. All estimates of probability are two-sided.

Results

Experiment 1

As is usual with this model,\textsuperscript{8,12} endothelial denudation modified the DNA content of the intima-media (Table 1). Initially on D1 and D2 as compared with D0, there was a significant decrease in DNA of 15.4% (95% CI: 5.2–25.7%, \( p=0.016 \)) and 19.5% (9.5–29.7%, \( p=0.0016 \)), respectively. This variation probably represents endothelial loss. Then, because of the proliferation of smooth muscle cells, the DNA content increased progressively to regain the initial value on D8 (\( p>0.25 \) as compared with D0) and to become significantly higher on D14: a mean increase of 39.6% (29–50%, \( p<10^{-4} \)).

For all study days (Table 1), the addition of a high insulin concentration to the incubation medium had no obvious effect on the rate of lipogenesis by intima-media (C versus Ins, \( p>0.05 \) by Mann-Whitney U test). By grouping the C and Ins values for each day, it appears that, in itself, the mitotic response of the intima-media to injury is accompanied by an increase in lipogenesis on D1, with a mean stimulation of 81% (29–133%, \( p=0.014 \)) compared with D0 and even more so on D2 (111% [59–163%], \( p=0.00051 \)). This metabolic stimulation is no longer apparent on D8 and D14.

Experiment 2

To further analyze the absence of insulin effect on the metabolism of the intima-media, we have focused on D2, when injury exerts its maximum stimulation on lipogenesis. Exposure of the aortic wall to increasing concentrations of insulin took place under nearly physiological pressure and flow rate: Overall means (\( n=46 \)) were, respectively, 90 mm Hg (95% CI: 1) and 32 ml/min (95% CI: 3) for measurements taken every minute during the 9-minute perfusion. The metabolism of the adventitia was also measured.

**Intima-media.** Again, the increase in lipogenesis induced by injury on D2 was apparent and even more pronounced than in experiment 1: Lipogenesis was 225% higher (130–320%, \( p=0.00011 \); overall ANOVA

**Table 1. Experiment 1: Lipogenesis by Intima-Media From Intact Aortas and Aortas at 1–14 Days After Injury**

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>DNA content (( \mu g ))</th>
<th>Lipid synthesis (pmol/( \mu g/min ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>D0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C*</td>
<td>4</td>
<td>52.1 (49.1–53.7)</td>
<td>0.425 (0.256–0.801)</td>
</tr>
<tr>
<td>Ins*</td>
<td>4</td>
<td>53.8 (52.7–62.7)</td>
<td>0.394 (0.286–0.777)</td>
</tr>
<tr>
<td>C+Ins†</td>
<td>8</td>
<td>53.8 (3.3)</td>
<td>0.471 (0.179)</td>
</tr>
<tr>
<td>D1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C*</td>
<td>4</td>
<td>46.8 (46.0–47.9)</td>
<td>0.663 (0.572–1.120)</td>
</tr>
<tr>
<td>Ins*</td>
<td>4</td>
<td>43.0 (39.4–50.7)</td>
<td>0.837 (0.591–1.153)</td>
</tr>
<tr>
<td>C+Ins†</td>
<td>8</td>
<td>45.4 (3.1)†</td>
<td>0.851 (0.188)§</td>
</tr>
<tr>
<td>D2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C*</td>
<td>4</td>
<td>43.3 (41.4–53.7)</td>
<td>1.314 (0.444–1.517)</td>
</tr>
<tr>
<td>Ins*</td>
<td>4</td>
<td>43.6 (29.2–47.5)</td>
<td>0.871 (0.413–1.199)</td>
</tr>
<tr>
<td>C+Ins†</td>
<td>8</td>
<td>43.2 (5.8)§</td>
<td>0.993 (0.354)§</td>
</tr>
<tr>
<td>D8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C*</td>
<td>4</td>
<td>56.0 (53.5–61.9)</td>
<td>0.446 (0.337–0.543)</td>
</tr>
<tr>
<td>Ins*</td>
<td>4</td>
<td>56.7 (47.5–64.9)</td>
<td>0.356 (0.297–0.404)</td>
</tr>
<tr>
<td>C+Ins†</td>
<td>8</td>
<td>56.6 (4.4)†</td>
<td>0.398 (0.067)‡</td>
</tr>
<tr>
<td>D14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C*</td>
<td>3</td>
<td>71.5 (67.3–85.0)</td>
<td>0.243 (0.201–0.255)</td>
</tr>
<tr>
<td>Ins*</td>
<td>4</td>
<td>74.2 (70.9–82.0)</td>
<td>0.258 (0.241–0.338)</td>
</tr>
<tr>
<td>C+Ins†</td>
<td>7</td>
<td>75.0 (5.9)†</td>
<td>0.256 (0.039)§</td>
</tr>
</tbody>
</table>

D0, day 0 (intact aortas); D1, D2, D8, D14 are days 1, 2, 8, and 14 after injury; C, control (no insulin); Ins, insulin added to the incubation medium (10^{-5} \( \mu \)m/\( ml \)).

\* Median (range); \( t \), Mean (95% confidence interval), with none of the differences between C and Ins groups being significant (Mann-Whitney U test) and the values on each given day grouped (C+Ins) for further analysis.

Comparisons with mean (C+Ins) at D0 (one-way analysis of variance and Bonferroni’s modified \( t \) test) and the values on each given day grouped (C+Ins) for further analysis.

\( p<0.05 \), \( p<0.02 \), \( p<0.002 \), [not significant (\( p>0.05 \), \( p<10^{-4} \)].
followed by Bonferroni's modified t test) in D2 aortas perfused without insulin than in D0 aortas: 0.458 pmol/μg DNA/min (95% CI: 0.115) versus 0.141 (95% CI: 0.069). Again, none of the tested concentrations of insulin in the perfusion medium exerted any stimulation on lipogenesis (Figure 1).

Adventitia. The results are opposite those for the intima-media. Injury itself had no influence on glucose metabolism, but insulin exposure did stimulate lipogenesis in a dose-dependent manner (Figure 1). The stimulation exerted by insulin was significant (Bonferroni's modified t test) at all concentrations tested: \( p<10^{-3} \) for \( 10^2 \) μunits/ml and \( p<10^{-6} \) for all higher concentrations \( (10^2-10^5 \) μunits/ml). A test of grouped linear correlations between the \( \log_{10} \) of insulin concentration in microunits per milliliter \( (x) \) and the lipogenic activity of the adventitia in picomoles per microgram per minute \( (y) \) gave a correlation coefficient (Pearson's \( r \)) of 0.874 (95% CI: 0.793–0.941, \( p<10^{-4} \)) with \( y=10.8+8.6x \) as the regression equation \( (y=a+bx) \). The 95% CIs were \(-17.6\) to \(-4.0\) for \( a \) and \( 6.6\) to \( 10.6\) for \( b \).

Discussion

Arterial injury with a balloon catheter provokes a well-documented mitotic reaction of smooth muscle cells. Both our experiments show that in its early phase (24–48 hours), the response to injury is associated with a definite increase in lipogenesis from glucose by the intima-media. Few previous studies have examined the metabolism of a recently injured artery. Rosenfeld et al,17 studying the intima-media of the rabbit aorta, found that 6 days after injury with a Fogarty catheter, incorporation of leucine (protein synthesis) was multiplied by 9.1, and syntheses of cholesterol and nonsaponifiable lipids from acetate were multiplied by 7.9 and 8.9, respectively; this increased metabolic activity persisted 1 year after endothelial injury.18 By studying the activity of various enzymes in the aortic homogenates from rabbits fed a normal diet or a diet enriched in cholesterol, Hajjar et al19 found that arterial injury 10 weeks after deendothelialization alters the metabolism of arterial cells and that regrowth of endothelium modifies these changes that favor cholesterol accumulation. None of these studies, however, directly examined lipid synthesis from glucose which, from our results, appears to be slightly and transiently increased (present on D1 and D2 but no longer on and after D8). Immediately after endothelial denudation, platelets...
adhere to the subendothelium. Activated platelets secrete growth regulatory peptides, which can also stimulate glucose metabolism in various cell lines: Epidermal growth factor (EGF) and type β-transforming growth factor (TGF-β) increase glycolysis; TGF-β stimulates the cellular capation of glucose, an effect that is enhanced by EGF and platelet-derived growth factor (PDGF); EGF and PDGF stimulate glycogen synthetase activity; PDGF also regulates glucose transporter expression. Interactions of platelets with the arterial wall could account in part for the transient stimulation of glucose metabolism by the intima-media, which is contemporaneous with the entry of smooth muscle cells into the mitotic cycle. This change characterizes the passage of muscle cells from the contractile to the synthetic or secretory phenotype. The main aim of our experiments was to assess whether phenotypic modulation modifies the metabolic response of arterial cells to an insulin exposure.

Experiment 1 confirms that exposure to a high concentration of insulin during a 1-hour incubation does not enhance lipogenesis from glucose by the intima-media and shows that arterial injury does not modify this lack of responsiveness. We found no metabolic response to insulin up to the 14th day after injury, when DNA synthesis by the intima-media has almost regained the low level of uninjured tissue (D0). As hydrodynamic forces may play a role in the metabolic response of the intima-media to insulin under extreme conditions (animals prepared by a 48-hour fast and a very high insulin concentration, $10^5 \mu$units/ml, in the perfusion medium), we chose in experiment 2 on D2 to further explore the question by exposing the isolated aorta, perfused under nearly physiological pressure and flow rate, to increasing concentrations of insulin ($10^2-10^5 \mu$units/ml). This experiment confirmed the stimulating effect of injury itself but did not show that the intima-media acquires any responsiveness to insulin. Even more, the intima-media when stimulated by injury seems to loose the weak insulin sensitivity that we have reported previously for the uninjured artery. However, because glucose is the primary metabolic target of insulin and because the main metabolism implied in atherogenesis is considered to be lipid metabolism, our results do not substantiate the view that insulin contributes to the development of arterial diseases (atherosclerosis, diabetic macroangiopathy) through its direct metabolic effects on arterial smooth muscle cells.


**KEY WORDS** • insulin • arterial injury • arterial metabolism • lipogenesis • atherosclerosis • diabetic macroangiopathy • smooth muscle phenotype
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L Capron and J Jarnet

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