Evidence That Acute Insulin Administration Enhances LDL Cholesterol Susceptibility to Oxidation in Healthy Humans

Alfredo Quiñones-Galvan, Anna Maria Sironi, Simona Baldi, Fabio Galetta, Ulisse Garbin, Anna Fratta-Pasini, Luciano Cominacini, Ele Ferrannini

Abstract—Increased free radical production and hyperinsulinemia are thought to play a role in experimental and human atherosclerosis, but the relation between the 2 abnormalities has not been studied. In 23 healthy volunteers, we measured the susceptibility of circulating low-density lipoprotein (LDL) cholesterol particles to in vitro copper sulfate oxidation (measured as the lag phase) and cell-mediated oxidative modification (measured as malondialdehyde generation in LDL during incubation with human umbilical vein endothelial cells), as well as the vitamin E content of LDL cholesterol at baseline and after 2 hours of physiological hyperinsulinemia (euglycemic insulin clamp). The lag time of LDL oxidation decreased from 108±3 and 107±3 minutes (at baseline and after 2 hours of saline infusion) to 101±3 minutes after 2 hours of clamping (P<0.0001). At corresponding times, cell-mediated malondialdehyde generation in LDL rose from 4.96±0.11 and 4.98±0.10 to 5.28±0.10 nmol/L (P=0.0006), whereas the LDL vitamin E content decreased from 6.78±0.06 and 6.77±0.06 to 6.64±0.06 μg/mg (P<0.04). The insulin-induced shortening of the lag phase was directly related to the decrement of vitamin E in LDL; furthermore, in subjects with higher baseline serum triglyceride levels, insulin induced a greater shortening of the lag phase than in subjects with low baseline triglycerides. We conclude that in healthy humans acute physiological hyperinsulinemia enhances the oxidative susceptibility of LDL cholesterol particles. This effect may have pathogenic significance for atherogenesis in insulin resistant states. (Arterioscler Thromb Vasc Biol. 1999;19:2928-2932.)

Key Words: insulin LDL cholesterol oxidation free radicals

Received April 22, 1999; revision accepted May 31, 1999.
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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

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algorithm. Before the start of the study and at timed intervals during the clamp, arterialized blood samples were obtained for the measurement of plasma glucose and insulin levels. At times 2 to 120 minutes of stabilization, saline was infused for 120 minutes (time-warming box (60°C) for arterialized blood sampling. After 30 minutes and then cooled and centrifuged at 2500 rpm for 10 minutes. The absorbance of the supernatant fraction was read at 532 nm, and the quantification was achieved by comparison with a standard curve of MDA equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetraethoxypropane. All measurements were made in duplicate.

Cholesterol and triglyceride levels in plasma were determined by Technicon AutoAnalyzer II methodology. Plasma glucose was measured by the glucose oxidase method with a glucose analyzer (Beckman Instruments), and insulin was measured by radioimmunoassay (Linco Research).

Data Analysis
Because previous studies have demonstrated that hepatic glucose production is fully suppressed during the second hour of an insulin clamp with the insulin dose used in the present studies, whole-body glucose utilization (insulin sensitivity) was calculated from the infusion rate of exogenous glucose after correction for changes in glucose levels in a distribution volume of 250 mL kg⁻¹. Glucose disposal rates were averaged over the second hour of the euglycemic clamp study, during which near-steady-state conditions prevailed, and expressed per kilogram of lean body mass.

Data are given as the mean±SEM; LDL oxidation parameters and vitamin E concentrations are given as median and interquartile range. Mean group values were compared with the use of the Wilcoxon signed rank test (2 groups) or Friedman test (3 groups). Simple and multiple regression analyses were carried out by standard methods.

Results
The plasma glucose and insulin concentrations at baseline and during the clamp are given in Table 1. The effect of insulin on serum lipid concentrations is summarized in Table 2. Saline infusion had no detectable effect on serum lipids. After 2 hours of euglycemic hyperinsulinemia, serum total cholesterol decreased by 2%; this change was due to a 22% decline in VLDL cholesterol levels and to a small (2%) decline in LDL cholesterol (P<0.01 for the difference between time 0 and 120 minutes). Serum total triglycerides decreased by 4%; this was the result of decrements in the triglyceride content of both the LDL (−7%) and VLDL fraction (−4%). Both cholesterol and triglycerides were essentially unchanged in the HDL fraction. Plasma free fatty acid concentrations decreased by 65±2% (average value during 2 hours of hyperinsulinemia).

Lag phase, MDA generation, and LDL vitamin E content were all interrelated at baseline (lag phase versus vitamin E, r=0.73, P<0.001; lag phase versus MDA, r=0.85, P<0.0001; and vitamin E versus MDA, r=0.69, P=0.0004). Furthermore, the lag phase was significantly shorter in subjects with higher mean blood pressure levels (r=0.42, P<0.05).

The lag phase of in vitro LDL oxidation was unchanged after 2 hours of saline infusion, whereas it decreased significantly after insulin infusion (Table 3). This effect of insulin

### TABLE 1. Characteristics of Study Subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean±SEM</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>47±2</td>
<td>30–60</td>
</tr>
<tr>
<td>BMI, kg · m⁻²</td>
<td>26.6±0.5</td>
<td>23.2–30.0</td>
</tr>
<tr>
<td>WHR, cm/cm</td>
<td>0.87±0.02</td>
<td>0.72–0.98</td>
</tr>
<tr>
<td>Mean blood pressure, mm Hg</td>
<td>106±3</td>
<td>90–128</td>
</tr>
<tr>
<td>Fasting plasma glucose, mmol/L</td>
<td>5.1±0.1</td>
<td>4.4–6.0</td>
</tr>
<tr>
<td>Steady-state plasma glucose, mmol/L</td>
<td>5.0±0.1</td>
<td>4.9–5.5</td>
</tr>
<tr>
<td>Fasting plasma insulin, pmol/L</td>
<td>70±10</td>
<td>18–196</td>
</tr>
<tr>
<td>Steady-state plasma insulin, pmol/L</td>
<td>671±40</td>
<td>455–1117</td>
</tr>
<tr>
<td>Insulin sensitivity, µmol · min⁻¹ · kg⁻¹</td>
<td>40±3</td>
<td>23–70</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; WHR, waist-to-hip ratio.

Subjects
We studied 23 healthy volunteers, 20 men and 3 women (Table 1). All subjects were free of intercurrent acute or chronic illness, were nonsmokers, were not engaged in competitive sports, and were not consuming vitamin supplements.

The study protocol was approved by the Institutional Ethics Committee, and the purpose, nature, and risks involved in the study were explained to all subjects before obtaining their written consent to participate.

Protocol
All subjects were studied at 9 AM after an overnight (10- to 12-hour) fast. A 20-gauge polyethylene catheter was inserted into an antecubital vein for infusion of test substances. A wrist vein was cannulated retrogradely with another catheter, and the hand was placed in a warming box (60°C) for arterialized blood sampling. After 30 minutes of stabilization, saline was infused for 120 minutes (time-control period). Next, insulin was infused at the rate of 7 pmol · min⁻¹ · kg⁻¹ (1 mU · min⁻¹ · kg⁻¹) while maintaining plasma glucose constant at the basal level by the euglycemic insulin clamp technique. Briefly, 4 minutes after starting exogenous insulin, an infusion of glucose (as a 20% D-glucose solution) was begun at an initial rate of 10 µmol · min⁻¹ · kg⁻¹; thereafter, the glucose infusion rate was adjusted every 5 minutes on the basis of on-line measurements of plasma glucose concentrations by using a computerized algorithm. Before the start of the study and at timed intervals during the clamp, arterialized blood samples were obtained for the measurement of plasma glucose and insulin levels. At times 120, 0, and 120 minutes, arterialized blood samples were drawn for the measurement of the following variables: lipid profile, vitamin E content in LDL, cell-mediated malondialdehyde (MDA) generation in LDL, and the susceptibility of LDL cholesterol to in vitro copper sulfate–induced oxidation.

Procedures
For lipoprotein separation, whole blood was collected into Vacutainer tubes (Becton Dickinson) containing EDTA (1 mg/mL) and immediately centrifuged at 2000 rpm for 20 minutes at 4°C. Plasma was stored at 4°C and processed for lipoprotein separation within 1 day. Lipoprotein was isolated by sequential ultracentrifugation in NaBr solution containing EDTA (1 mg/mL) and stored at 4°C. To minimize the possibility of LDL oxidation during isolation, all solutions used in this process were deoxygenated by argon bubbling. LDL was stored under nitrogen at 4°C in a sterile dark environment and processed within 1 day.

Susceptibility of LDL to oxidation by copper was evaluated by measuring the length of the lag phase on the basis of the development of fluorescence during copper-catalyzed LDL oxidative modification, as previously described. High-performance liquid chromatography was used to measure vitamin E content in LDL cholesterol, as previously described. Cell-mediated LDL oxidative modification was also determined. Human umbilical vein endothelial cells were isolated as described and used at passages 2 to 4. LDL oxidation was prepared by adding 1.5 mL of serum-free F-12 medium containing 200 µg/mL of protein to each well of human umbilical vein endothelial cells and incubating at 37°C for 24 hours. The extent of LDL oxidation was determined by measuring the concentration of thiobarbituric acid–reactive substances, as described. Briefly, aliquots of the incubation mixture containing 200 µg LDL were removed and added to tubes containing 0.05 mL of 2% butylated hydroxytoluene, 2 mL of 0.67% thiobarbituric acid, and 10% trichloroacetic acid (2:1). The tubes were heated at 100°C for 10 minutes and then cooled and centrifuged at 2500 rpm for 10 minutes. The absorbance of the supernatant fraction was read at 532 nm, and the quantification was achieved by comparison with a standard curve of MDA equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetraethoxypropane.
was seen in 20 of 23 subjects (Figure). Likewise, MDA generation and LDL vitamin E content were not altered by saline infusion, whereas MDA generation was significantly increased and LDL vitamin E content was significantly decreased at the end of the clamp (Table 3). The observed changes in oxidative parameters were consensual. Thus, the reduction in lag phase after insulin was directly related to the decrement of LDL vitamin E content ($r=0.55, P<0.01$) and tended to be related to the rise in MDA generation ($r=0.36, P=0.09$). A greater insulin-induced reduction in lag phase was associated with higher fasting triglyceride levels both in whole plasma and in the VLDL fraction ($r=0.46, P<0.03$ for both). When the study group was subdivided according to the median serum triglyceride concentration, the insulin-induced decrease in lag phase was directly related ($P=0.01$) to baseline serum triglycerides in the 11 subjects with higher triglyceride levels (2.04 $\pm$ 0.28 mmol/L) but not in those with lower triglyceride levels (0.84 $\pm$ 0.08 mmol/L, $P=NS$). In contrast, none of the insulin-induced changes in oxidative parameters was related to insulin sensitivity or any other anthropometric or metabolic variable.

**Discussion**

In the present study, acute in vivo insulin administration increased the susceptibility of LDL cholesterol to both copper-induced and cell-mediated oxidation. This effect of insulin was consistent and specific (no effect of saline infusion). The increased susceptibility of the LDL cholesterol particles to oxidation was closely related to a decrease in their vitamin E content but not to the insulin sensitivity of glucose metabolism. Using a different probe (in vitro incubation of LDL with alanine) to estimate the amount of partially oxidized LDL present in vivo, Carantoni et al$^{24}$ reported that in nondiabetic subjects the presence of insulin resistance of glucose metabolism was associated with increased serum levels of partially oxidized LDL. Although we cannot exclude that this was also the case in our subjects, we found that in vivo insulin administration enhanced LDL oxidizability similarly in insulin-sensitive and -resistant individuals. This suggests that dyslipidemia, especially hypertriglyceridemia, is associated both with insulin resistance of glucose metabolism and with increased partially oxidized LDL particles. However, the pathway through which insulin generates

| TABLE 2. Serum Lipid Concentrations in Fasting State and After Euglycemic Hyperinsulinemia |
|---------------------------------|-----------------|-----------------|------|
|                                | Baseline        | Time Control    | Insulin | $P^*$ |
| Total cholesterol, mmol/L      | 5.14 $\pm$ 0.15 | 5.12 $\pm$ 0.15 | 5.02 $\pm$ 0.15 | 0.01 |
| LDL cholesterol, mmol/L        | 3.37 $\pm$ 0.12 | 3.40 $\pm$ 0.12 | 3.33 $\pm$ 0.12 | 0.08 |
| VLDL cholesterol, mmol/L       | 0.46 $\pm$ 0.07 | 0.43 $\pm$ 0.06 | 0.37 $\pm$ 0.05 | <0.01|
| HDL cholesterol, mmol/L        | 1.42 $\pm$ 0.07 | 1.38 $\pm$ 0.07 | 1.38 $\pm$ 0.07 | n.s. |
| Total triglycerides, mmol/L    | 1.41 $\pm$ 0.18 | 1.38 $\pm$ 0.18 | 1.34 $\pm$ 0.19 | 0.001|
| LDL triglycerides, mmol/L      | 0.29 $\pm$ 0.01 | 0.28 $\pm$ 0.01 | 0.27 $\pm$ 0.01 | <0.0001|
| VLDL triglycerides, mmol/L     | 0.93 $\pm$ 0.16 | 0.92 $\pm$ 0.16 | 0.88 $\pm$ 0.17 | 0.002|
| HDL triglycerides, mmol/L      | 0.20 $\pm$ 0.02 | 0.20 $\pm$ 0.02 | 0.19 $\pm$ 0.03 | 0.06 |
| Free fatty acids, mmol/L       | 0.59 $\pm$ 0.04 | 0.59 $\pm$ 0.04 | 0.10 $\pm$ 0.01 | <0.0001|

Values are mean $\pm$ SEM. *By Friedman test.

| TABLE 3. Parameters of LDL Oxidizability in Fasting State and After Euglycemic Hyperinsulinemia |
|---------------------------------|-----------------|-----------------|------|
|                                | Baseline        | Time Control    | Insulin | $P^*$ |
| Vitamin E contents, $\mu g/mg$ | 6.77 (0.35)     | 6.78 (0.48)     | 6.69 (0.35) | 0.04 |
| Lag phase, min                 | 101 (29)        | 101 (30)        | 94 (20)  | <0.0001|
| MDA, nmol/L                    | 5.06 (0.85)     | 5.11 (0.79)     | 5.30 (0.70) | 0.0003|

Values are median, with interquartile range in parentheses. Entries are vitamin E contents in the LDL fraction, lag phase of copper-induced LDL oxidation, and medium malondialdehyde concentrations during cell-mediated LDL oxidation.

**By Friedman test.*
oxygen-reactive species may be independent of the glucose pathway or may already be saturated at the plasma insulin concentrations (∼700 pmol/L) that we used in these clamp studies.

It must be noted that insulin promotes LDL cholesterol uptake by upregulating LDL receptors. If large, more buoyant LDL particles were preferred to small, dense LDL molecules in such a process, insulinization would be associated with an enrichment of plasma with small, dense LDL particles, intrinsically more susceptible to oxidation. In the present study, insulin did cause a small decrease in serum LDL concentrations; in addition, the possibility that insulin may cause an in vivo selection of lipoprotein has been recently documented in studies measuring LDL size after an oral glucose load.

Higher serum triglyceride levels are generally associated with a predominance of small, dense LDL. In the present study, the lack of correlation between fasting triglyceride levels and LDL lag phase could be due to the relatively small sample size or could reflect the fact that very few subjects were hypertriglyceridemic (only 3 subjects >2.3 mmol/L) or could be a combination of these 2 factors. On the other hand, the presence of higher triglycerides, in whole serum or the VLDL fraction, was associated with an enhanced effect of insulin, suggesting that higher triglycerides do signal an increased susceptibility of LDL to oxidative stimuli. Insulin also induced a small decrement (4% on average) in circulating triglyceride levels. Thus, one could surmise either that the insulin-induced shortening of the lag phase would have been even larger had the triglycerides remained unchanged or that the kinetics of the insulin effect was faster than any secondary effect mediated by changes in triglycerides. In general, the nature of the relation between triglycerides and LDL oxidizability is imperfectly understood. On the one hand, when plasma triglycerides are reduced with intensive exercise and dieting or by fibrate therapy, in vitro LDL cholesterol oxidizability is reduced. On the other hand, recent studies have shown that if triglyceride levels are acutely increased (by an infusion of Intralipid [triglyceride emulsion]), the susceptibility of LDL cholesterol to oxidation is decreased rather than increased. From these results, it has been suggested that enriching the LDL molecule with triglycerides reduces its oxidizability, whereas the subsequent hydrolysis of triglycerides within LDL can lead to atherogenic changes. Collectively, these data suggest that one should distinguish between the acute and chronic effects of lipid exchange between lipoprotein particles on their susceptibility to oxidation. Clearly, additional work is needed to gain insight into these mechanisms.

The insulin effect observed in the present studies was small in size (averaging 7%). However, the experiment was designed to test whether insulin alone (ie, without hyperglycemia) at physiological concentrations had any acute effects on LDL oxidizability. On the other hand, chronic hyperinsulinemia, such as prevails in insulin-resistant individuals, may translate into a larger insulin pro-oxidant effect. In this respect, it is relevant that treatment of type 2 diabetic patients with troglitazone, a thiazolidinedione compound with an α-tocopheryl moiety, leads to an improvement in insulin sensitivity (and plasma insulin levels) and a reduction in LDL susceptibility to oxidation. The extent to which the ex vivo measurements of LDL cholesterol oxidation reflect the in vivo oxidative damage of lipoproteins has been generally uncertain. Although this uncertainty has not prevented a wide application of the methodology to a number of clinical conditions, the presence of oxidized LDL in vivo has recently been demonstrated in human serum by NMR spectroscopy. Importantly, the results of this assay agreed well with the simultaneously measured in vitro LDL oxidizability, indicating that the latter does reflect a biological phenomenon.

A series of studies using human aortic intimal cells was reviewed by Sobenin et al, who demonstrated that sera from type 2 diabetic patients show increased atherogenic properties, as measured by the ability of LDL cholesterol to accumulate in these cells. The modifications to the LDL molecule included nonenzymatic glycosylation and loss of sialic acid. Some of these atherogenic modifications of the LDL cholesterol particle can be induced by an excessive activity of free radicals. In vivo, hyperglycemia has been invariably regarded as the principal culprit of the increased free radical production observed in type 2 diabetic patients. In line with this notion, in type 2 diabetic patients the total plasma antioxidation capacity, erythrocyte lipid peroxidation activity, the susceptibility of LDL cholesterol to oxidation, and the excretion of oxidized products of arachidonic acid are all increased, in proportion to the severity of hyperglycemia. Even in nondiabetic subjects, the relative hyperglycermia induced by an oral glucose tolerance test stimulates the production of free radicals. Furthermore, increased reactive oxygen species activity has been documented in hyperinsulinemic nonhyperglycemic conditions, such as obesity, essential hypertension, smoking, and dyslipidemia. Also, a long latency between the histological evidence of atherosclerosis and the onset of hyperglyceremia in type 2 diabetes is the rule. Atherogenesis is likely to set in when the only abnormality (in future type 2 diabetic or hypertensive patients) is insulin resistance/hyperinsulinemia and its metabolic correlates. Activation of peroxidation (lipid-conjugated dienes and MDA) is already present in the initial lesion of atherosclerosis, the fatty streak.

In conclusion, insulin at physiological doses is associated with increased LDL peroxidation independent of the presence of hyperglycemia. This result supports the possibility that insulin resistance may be implicated in the genesis of the early atherosclerotic lesions (and/or the progression of atherosclerosis) through the attendant hyperinsulinemia.

References


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Arterioscler Thromb Vasc Biol. 1999;19:2928-2932
doi: 10.1161/01.ATV.19.12.2928

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