Oral Insulin Supplementation Attenuates Atherosclerosis Progression in Apolipoprotein E–Deficient Mice

Raanan Shamir, Naim Shehadeh, Mira Rosenblat, Orly Eshach-Adiv, Raymond Coleman, Marielle Kaplan, Shadi Hamoud, Sophie Lischinsky, Tony Hayek

Objective—The role of insulin in atherosclerosis progression in diabetes is uncertain. We examined the effects of oral insulin supplementation on atherogenesis in apolipoprotein E–deficient (E0) mice.

Methods and Results—One-month-old male E0 mice were orally supplemented with human insulin (0.1, 0.5, and 1 U/mL) or placebo for 3 months. At the end of the study, serum and macrophage oxidative stress and atherosclerosis progression were studied. Insulin reduced lesion size by 22% to 37% (P<0.05) in all study groups. Lipid peroxides serum levels were 18% lower (P<0.01), and serum paraoxonase activity was 30% higher (P<0.01) in mice supplemented with 1.0 U/mL insulin compared with controls. Insulin reduced mouse peritoneal macrophage (MPM) lipid peroxides content and superoxide anion release by up to 44% and 62%, respectively (P<0.01). In addition, oral insulin reduced MPM cholesterol content and cholesterol biosynthesis by up to 36% and 53%, respectively (P<0.01). In vitro incubation of E0 mice MPM with increasing insulin concentrations (0 to 100 μU/mL) resulted in a dose-dependent reduction of cholesterol synthesis by up to 66% (P<0.05).

Conclusions—In E0 mice, oral insulin supplementation attenuates the atherosclerotic process. This may be attributable to insulin-mediated reduction of oxidative stress in serum and macrophages as well as reduction in macrophage cholesterol content. (Arterioscler Thromb Vasc Biol. 2003;23:104-110.)

Key Words: oral insulin atherosclerosis oxidative stress paraoxonase macrophages

Atherosclerosis is the major cause of morbidity and mortality in the western world, and diabetes mellitus is one of the major risk factors for atherosclerosis development.1 The role of insulin, however, in atherosclerosis progression is uncertain. Hyperinsulinemia is considered to be an independent risk factor for atherosclerosis development,1,2 but there are some lines of evidence suggesting a protective role for insulin.3 Recently, we demonstrated that oral insulin supplementation increased insulin serum levels in Balb-C mice.4 In the present study, we evaluated the effect of oral insulin supplementation on atherosclerosis progression in apolipoprotein E–deficient (E0) mice. The E0 mice model is widely used to study atherogenesis, because these mice develop severe hypercholesterolemia and accelerated atherosclerosis and their atherosclerotic lesion composition is similar to human atherosclerotic lesion.5 In addition, the E0 mice are under oxidative stress, and their serum lipids, as well as their harvested macrophages, contain oxidized lipids.6,7 Similarly, diabetic patients are under oxidative stress and prone to develop atherosclerosis.8 Thus, we questioned whether insulin supplementation to these mice has an effect on the oxidative status of serum and macrophages. Furthermore, because macrophages play an essential role in the development of atherosclerosis9,10 and cholesterol accumulation in macrophages leads to foam cell formation,9,10 we evaluated the effects of insulin supplementation on cholesterol metabolism in macrophages.

In the present study, we have demonstrated for the first time that oral insulin supplementation to the atherosclerotic E0 mice resulted in a significant reduction in aortic atherosclerotic lesion size, and this may be mediated by reduction of serum and macrophage oxidative status as well as reduction in macrophage cholesterol biosynthesis.

Methods

Materials

Cytochrome C from horse heart, SOD from bovine erythrocytes, and BSA were purchased from Sigma. DMEM, HBSS, RPMI, PBS, FCS, streptomycin, and penicillin were purchased from Biological Industries. [3 H]-acetate sodium salt or [14 C]-mevalonate was purchased from Amersham.

Mice Studies

The study conformed with the Guide for Care and Use of Laboratory Animals published by the United States National Institutes of Health.

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From the Pediatric Gastroenterology and Nutrition Unit (R.S., O.E.A.) and Juvenile Diabetes Unit (N.S.), Meyer Children’s Hospital of Haifa; Department of Medicine E (S.H., T.H.) and Department of Biochemistry (S.L.), Rambam Medical Center; and Lipid Research Laboratory (M.R., M.K., T.H.) and Department of Anatomy and Cell Biology (R.C.), Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel.

Correspondence to Raanan Shamir, MD, Division of Pediatric Gastroenterology and Nutrition, Meyer Children’s Hospital of Haifa, Rambam Medical Center, POB 9602, Haifa 31096, Israel. E-mail shamirr@netvision.net.il

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E\textsuperscript{\texttt{0}} mice were generously provided by Dr Jan Breslow, Rockefeller University, New York.

Human insulin (Humulin R, Eli Lilly) was given to 4-week-old male E\textsuperscript{\texttt{0}} mice in their drinking water for 3 months. Mice were supplemented with 0.1 U/mL (n=6), 0.5 U/mL (n=6), or 1 U/mL (n=6) of insulin. The placebo group received only water (n=6). All animals were fed an identical chow diet.

### Mice Weight and Blood Pressure

Mice body weights and blood pressure were recorded before and at the end of the treatment period in all animals, as described previously.\textsuperscript{11} All blood pressure measurements were repeated 5 times for each animal.

### Blood Glucose and Insulin

At the end of the study period, blood was collected from the retroorbital plexus. Blood glucose was determined using a Glucometer (Elite, Kyoto Daiichi Kagaku Co) and by using an enzymatic kit (Roche Diagnostics). Insulin concentrations were determined by radioimmunoassay with a commercial kit (DiaSorin). The principle of the assay is based on the competition between labeled insulin and insulin contained in standards or specimens to be assayed for a fixed and limited number of antibody binding sites. The amount of labeled insulin bound to the monoclonal antibody (which is 100% specific for human insulin) is inversely related to the amount of unlabelled human insulin present in the sample. The assay sensitivity, defined as the apparent concentration of analyte that can be distinguished from the zero standard, is below 4 \mu U/mL at 95% confidence limit. The assay accuracy has been checked by the dilution and recovery tests.

### Serum Lipids

Serum total cholesterol and triglycerides were measured by enzymatic spectrophotometric determination.\textsuperscript{12, 13} Direct measurement of high-density lipoprotein cholesterol was performed using polyethylene glycol-modified enzymes as previously described.\textsuperscript{14}

### Serum Lipid Peroxidation

Basal serum lipid peroxidation state was determined by measuring lipid peroxides level.\textsuperscript{15} In this assay, the capacity of peroxide to convert iodide to iodine is determined photometrically at 365 nm.\textsuperscript{15}

### Serum Paraoxonase Activity

Arylesterase and paraoxonase activities were measured in serum samples spectrophotometrically using phenylacetate or paraoxon as the substrate, respectively.\textsuperscript{11} One unit of arylesterase or paraoxonase activity is equal to 1 \mu mol or 1 mmol of substrate hydrolyzed/mL per min, respectively.\textsuperscript{11}

### Histomorphometry of Atherosclerotic Lesions

At euthanasia, the aortic arch was rapidly dissected out and fixed in 3% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) before postfixation in 1% aqueous osmium tetroxide and embedding in epoxy resin as described in detail previously.\textsuperscript{11} Histomorphometry of areas occupied by atherosclerotic lesions was performed in thin transverse sections of the aortic arch using an Olympus Cux-2 image analysis system.\textsuperscript{11}

### Isolation of Mouse Peritoneal Macrophages

Mouse peritoneal macrophages (MPMs) were harvested 4 days after intraperitoneal injection of thioglycollate (72 \mu g/mouse).\textsuperscript{2} The MPMs from 6 mice in each group were pooled into two pools. The cell suspension (10⁶/mL) was dispensed into 35-mm dishes and incubated (5% CO\textsubscript{2}, 95% air) for 2 hours. Cells were washed and additionally incubated under similar conditions.

### Macrophage Lipid Peroxides

The cell monolayers (3×10⁶) were washed with PBS and lipid extracted with hexane; isopropanol (3:2, vol/vol). The upper hexane phase was collected and dried under nitrogen. NaOH 0.1N was added to the cells for protein determination by the Lowry method.\textsuperscript{37} The amount of lipid peroxides was determined in the dried samples.\textsuperscript{15}

### LDL Oxidation by Macrophages

LDL was isolated from plasma of fasted normolipidemic human volunteers by discontinuous density gradient ultracentrifugation.\textsuperscript{16} LDL (100 \mu g of protein/mL) was incubated without or with MPM (2×10⁶) for 6 hours at 37°C in the presence of 5 \mu mol/L CuSO\textsubscript{4} in RPMI medium (without phenol red). The extent of LDL oxidation was measured as previously described.\textsuperscript{7}

### Superoxide Anion Generation by Macrophages

The production of the superoxide anion (O\textsuperscript{2-}) by MPM (2.5×10⁶/well) with no addition of any stimulator was measured as the SOD inhibitable reduction of acetyl ferricytochrome C.\textsuperscript{7}

### Macrophage Cholesterol Mass

Macrophage total cholesterol was extracted from the cells (3×10⁶), and its amount in the dried samples was determined using the CHO (CHOD-PAP) Kit (Roche Diagnosticum, Mannheim, Germany). A cholesterol standard (Sigma) was used for the calibration curve.

### Macrophage Cholesterol Biosynthesis

Cells (3×10⁶) were incubated for 18 hours at 37°C with [\textsuperscript{3}H]-acetate (1 \mu Ci/mL) or with [\textsuperscript{14}C]-mevalonate (2.5 \mu Ci/mL). Cellular lipids were extracted, separated by thin layer chromatography on silica gel plates, and developed in hexane:ether:acetic acid (130:30:1.5, vol/vol/vol). Unesterified cholesterol spots were visualized by iodine vapor, using standard for identification, scraped into scintillation vials, and counted in a \textbeta-counter.

### Statistical Analysis

Statistical analysis was performed with SPSS 9.0 software (SPSS Inc.). Data are expressed as mean±SD for each group. Comparisons for significant differences between two groups were performed by Student’s t test. For comparison of 4 groups, ANOVA was used. For single parameters, the x² test was used.

### Results

#### In Vivo Studies

**Effect of Oral Insulin on Atherosclerotic Lesion Development**

The effect of oral insulin supplementation on atherosclerosis development was analyzed in E\textsuperscript{\texttt{0}} mice supplemented with 3 different dosages of insulin for 3 months in comparison with placebo-treated mice. Insulin supplementation significantly (P<0.05) reduced the mean atherosclerotic lesion area by 37%, 28%, and 22% in mice supplemented with 0.1, 0.5, and 1.0 U/mL of insulin, respectively, compared with placebo-treated mice (Figure 1A). In addition, the mean number of lesions per mouse in the group supplemented with 1.0 U/mL of insulin was 48% lower than the placebo group and the other supplemented mice (P<0.01, Figure 1B). No major differences were seen in the histopathology of the 4 groups.

**Effects of Oral Insulin Supplementation on Weight, Blood Pressure, Serum Glucose, Insulin, and Lipid Levels**

Baseline weight and blood pressure were similar in all study groups, and oral insulin supplementation had no significant effect on these parameters (Table). As shown in the Table, insulin was found in the serum of all study groups, and in the group receiving 1.0 U/mL, it was significantly higher (P<0.05) than in the other study groups, indicating that the oral insulin was indeed absorbed. Serum glucose levels were
the amount of lipid peroxides in insulin-supplemented mice MPM. Oral insulin supplementation to E° mice resulted in a reduction in MPM lipid peroxides by 14%, 44%, and 41% in mice supplemented with 0.1, 0.5, or 1 U/mL insulin, respectively, compared with the level of lipid peroxides in placebo-treated mice MPM (Figure 2A, \( P < 0.01 \)). Accumulation of oxidized lipids in the macrophages affects their capability to release superoxide anions and to oxidize LDL.\(^7\) Indeed, the reduced lipid peroxide content was accompanied by a dose-dependent reduction in superoxide anion release from MPM by 49%, 55%, and 62% in mice supplemented with 0.1, 0.5, and 1 U/mL insulin, respectively, compared with placebo-treated mice (Figure 2B, \( P < 0.01 \)). LDL oxidation after 6-hour incubation in the presence of 5 \( \mu \)mol/L CuSO\(_4\) with MPM harvested from E° mice supplemented with 1 U/mL was lower by 15% compared with LDL oxidation by placebo-treated mice MPM (18.6 ± 1.5 vs 21.9 ± 2.1 nmol MDA/mg LDL protein, respectively, not statistically significant).

**Effects of Oral Insulin Supplementation on Macrophage Cholesterol Metabolism**

Accumulation of cholesterol in macrophages converts macrophages into foam cells and is the hallmark of atherosclerotic lesion formation.\(^9,10\) Thus, we next measured the amount of total cholesterol in insulin-treated mice MPM. Oral insulin supplementation to E° mice resulted in a dose-dependent reduction in MPM cellular cholesterol content (Figure 3A). Whereas in mice supplemented with 0.1 U/mL insulin no significant effect on MPM cholesterol levels was observed, there was a significant reduction (\( P < 0.01 \)) in MPM cholesterol content by 31% and 36% in mice supplemented with 0.5 or 1 U/mL of insulin, respectively, compared with the amount of cholesterol in placebo-mice MPM (Figure 3A).

To examine if decreased cholesterol biosynthesis may be the cause for reduced cholesterol mass, the ability of macrophages harvested from the insulin-supplemented E° mice to synthesize cholesterol from \( [\text{3H}]\)-acetate was next determined. As seen in Figure 3B, there was a significant reduction (\( P < 0.01 \)) in MPM cholesterol biosynthesis (by 42% to 53%) in mice supplemented with 0.1 to 1 U/mL of insulin compared with placebo-treated mice MPM (Figure 3B).

**In Vitro Studies**

**Effects of Insulin on Macrophage Oxidative Stress In Vitro: Concentration Study**

To assess the direct effects of insulin on macrophage oxidative stress, MPMs from 4-month-old E° mice were incubated for 18 hours with increasing concentrations of insulin (0 to 100 \( \mu \)U/mL). As can be seen in Figure 4A, there was a dose-dependent decrease in MPM lipid peroxide content (up to 46%) after adding 1 \( \mu \)U/mL insulin compared with lipid peroxides content in nontreated MPM. However, the decrease was not statistically significant, and higher insulin concentrations were less effective (adding 10 or 100 \( \mu \)U/mL of insulin reduced MPM lipid peroxides content only by 12% and 20%, respectively, Figure 4A). The amount of superoxide anions released from MPM after their incubation with increasing insulin concentrations (0 to 100 \( \mu \)U/mL) was then measured.
biosynthesis from [14C]-mevalonate was seen only at concentrations of 1.0 to 100 μU/mL of insulin (Figure 5B, \( P<0.01 \) between each concentration).

**Discussion**

Supplementation of oral insulin to E0 mice resulted in decreased aortic atherosclerotic mean plaque area and reduced the number of atherosclerotic lesions. These effects of oral insulin were associated with reduced oxidative stress both in serum and in MPM as well as reduced cholesterol biosynthesis in macrophages.

Hyperinsulinemia is an independent cardiovascular risk factor. However, the contribution of insulin itself to the atherosclerotic process is unknown, because insulin resistance could be responsible for the increased risk rather than the compensatory hyperinsulinemia observed with insulin resistance. As part of insulin resistance, there is increased lipid peroxidation attributable to increased production of free radicals, hyperglycemia increases intracellular reactive oxygen species, and postprandial hyperglycemia may be a risk factor for cardiovascular disease in diabetic patients.

There are some lines of evidence suggesting that insulin may have beneficial effects on the cardiovascular system. Insulin causes vasodilation in healthy humans and improves endothelial relaxation through nitric oxide production. Insulin attenuates the vascular smooth muscle cell response to vasoactive substances and may decrease large arteries’ stiffness. Insulin has been shown to lower blood pressure in some studies, whereas in another study it had no effect.

In contrast, insulin may be related to increased risk for atherosclerosis either secondary to obesity, hyperlipidemia, hypertension, hyperuricemia, and the use of oral contraceptives or through inhibition of lipolysis and by induction of vascular smooth muscle cell proliferation.

Findings in animal models are few and inconsistent. The administration of subcutaneous insulin to rats caused an increase in triglyceride content in the aorta and in intimal thickening. In rabbits fed with an atherogenic diet, the administration of subcutaneous insulin significantly reduced plasma lipid levels, but continued administration of insulin during atherosclerosis development resulted in reinforcement of the lipemia with aggravation of atherosclerosis.
ingly, in that study, after switching to a normal diet at the age of 2 months, insulin accelerated the reduction in hypercholesterolemia. In a similar study in rabbits, no effects on atherosclerosis or on lipid serum levels were demonstrated. In contrast, we were able to demonstrate in the present study that adding insulin at 1 U/mL to the drinking water of the atherosclerotic E0 mice for 3 months significantly reduced the number of atherosclerotic lesions per mouse, and all insulin concentrations resulted in significant reduction in mean atherosclerotic lesion area. Oral insulin supplementation resulted in a dose-dependent increase in serum insulin concentrations. However, portal insulin levels may be several times higher than those found in the systemic circulation.

LDL oxidation is important in atherosclerosis progression, and in E0 mice, increased plasma lipoprotein lipid peroxidation was documented. In our study, insulin supplementation to E0 mice caused a significant reduction in serum lipid peroxides and a significant increase in serum paraoxonase (PON1) activities. The increase in PON1 activity may explain the reduced serum oxidative stress, because it was shown that paraoxonase reduces lipid peroxides levels in oxidized lipoproteins. Reduced serum PON1 activity is seen in atherosclerotic patients, including diabetic patients, in insulin-deficient (streptozotocin-induced) diabetic rats, in E0 mice, and in rabbits fed an atherogenic diet. Thus, similar to its effect on E0 mice, insulin supplementation may increase PON1 activity in diabetes.

It was recently shown that E0 mice macrophages, which contain oxidized lipids, release increased amounts of superoxide anions and oxidize LDL at an enhanced rate. In that study, the reduction of oxidative stress by antioxidants supplementation reduced the amount of lipid peroxides in macrophages and their ability to oxidize LDL. In the present study, insulin supplementation to the E0 mice resulted in the reduction in macrophage lipid peroxides content in association with reduction in the cell’s ability to release superoxide anions and to oxidize LDL. The reduction in macrophage oxidative stress may again be explained by increased paraoxonase activity, because paraoxonase was shown to reduce specific oxidized lipids in macrophages and in atherosclerotic lesion. In addition, the decreased oxidation observed in our study could be the result of increased reduced glutathione (GSH) concentration. GSH is the major antioxidant in cells, and it has been shown that on incubation of cells with insulin,
the amount of GSH was significantly increased. In addition, it was shown that increasing GSH cellular content in the cells reduced their capability to release superoxide anions and to oxidized LDL. Furthermore, in diabetic rat ventricular myocytes, GSH homeostasis is functionally coupled to insulin signaling.

To assess the direct role of insulin on macrophages, we performed in vitro experiments. By incubating E0 mice macrophages with increasing concentrations of insulin (0 to 100 μU/mL), we observed no significant trend toward reduced oxidative stress in macrophages. First, this could be the result of differences between the time period macrophages acutely were exposed to insulin in the in vitro studies compared with the chronic exposure in the ex vivo studies. Second, the intracellular concentrations of insulin may have been different in the ex vivo versus the in vitro studies. Third, oral insulin supplementation may have triggered other, not yet identified, antioxidative pathways that could explain the lack of a significant effect in the in vitro studies.

Accumulation of cholesterol in macrophages is an essential step in atherosclerosis progression, and insulin was found to regulate cholesterol metabolism in macrophages. In our study, we demonstrated that insulin inhibited cholesterol synthesis in peritoneal macrophages in a dose-dependent manner, both in the ex vivo and in the in vitro studies. Thus, the reduced macrophage cholesterol content in insulin supplemented mice may be attributable to the inhibition of cholesterol synthesis induced by insulin. This is in agreement with other observations that insulin therapy depresses the rate of cholesterologenesis in patients with non–insulin-dependent diabetes mellitus. Also, increased cholesterol synthesis and 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase activity were previously demonstrated in MPM from insulin-deficient mice. In that study, insulin addition resulted in reduced HMGCoA-reductase activity and reduced cholesterol biosynthesis. However, in the present study, mevalonate blocked the inhibitory effect of insulin on cholesterol biosynthesis only at low insulin concentrations, suggesting that the inhibition of cholesterol biosynthesis by insulin may involve other, still undetermined, pathways.

In summary, in E0 mice, oral insulin supplementation attenuates the atherosclerotic process. It is conceivable that the effects of oral insulin supplementation on the atherosclerotic process are mediated via effects on oxidative stress in

Figure 4. Effects of increasing concentrations of insulin on macrophage oxidative stress in vitro. MPM were harvested from 4-month-old E0 mice. The cells were incubated for 18 hours at 37°C with increasing insulin concentrations (0, 0.01, 0.1, 1, 10, and 100 μU/mL) in DMED + 5%FCS after cell wash. A, Cellular lipids were extracted using hexane:isopropanol (3:2, vol/vol), for lipid peroxides determination. B, Amount of superoxide anion released from the cells was determined after 1 hour of incubation at 37°C. C, Cells were additionally incubated for 6 hours with LDL (100 μg of protein/mL) in the presence of 5 μmol/L CuSO4. The extent of LDL oxidation was determined by the TBARS assay.

Figure 5. Effects of increasing concentrations of insulin on macrophage cholesterol metabolism in vitro. MPMs were harvested from 4-month-old E0 mice. The cells were incubated with increasing insulin concentrations (0, 0.01, 0.1, 1, 10, and 100 μU/mL) for 18 hours at 37°C in DMED + 5%FCS. The cells were then washed and additionally incubated with 18 hours at 37°C with [3H]-acetate (A) or with [14C]-mevalonate (B) for cholesterol biosynthesis determination. Results are given as mean ± SD of 3 different experiments, each performed in triplicate, *P < 0.01 (vs 0 concentration), &P < 0.05 (vs 0.01 μU/mL), #P < 0.01 (vs 0.1 μU/mL), @P < 0.01 (vs 1 μU/mL), †P < 0.01 (vs 10 μU/mL).
serum and in macrophages as well as effects on macrophage cholesterol content and biosynthesis.

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