Insulin Suppresses HDL-Mediated Cholesterol Efflux from Macrophages Through Inhibition of Neutral Cholesteryl Ester Hydrolase and ATP-Binding Cassette Transporter G1 Expressions

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**Aims:** We studied the effect of insulin on HDL-mediated cholesterol efflux from macrophages. The potential involvement of cholesteryl ester hydrolysis and membrane cholesterol transport was also addressed.

**Methods:** Human monocyte-derived THP-1 cells were developed into macrophages. Cholesterol efflux was measured by incubating macrophages, labeled with $[^3H]$-cholesterol, with HDL for 24 h. The cells were treated with insulin (0−500 nM) for 30 min prior to the addition of HDL. To investigate the molecular mechanisms of the effect of insulin, the expressions of neutral cholesteryl ester hydrolase (nCEH) and ATP-binding cassette transporter (ABC) G1 were analyzed.

**Results:** Insulin inhibited, in a concentration-dependent manner, HDL-mediated cholesterol efflux from macrophages. Insulin also inhibited the enzyme activity of nCEH and its mRNA and protein expression in cells. Insulin also suppressed the expressions of mRNA and protein for ABCG1.

**Conclusions:** Insulin inhibits HDL-mediated cholesterol efflux from macrophages, which may result from the suppression of nCEH and ABCG1 expressions. Our findings show part of the potential molecular mechanism of atherogenesis in type 2 diabetes with hyperinsulinemia.

*J Atheroscler Thromb, 2010; 17:1183-1189.*

**Key words:** Cholesterol efflux, HDL, Insulin, nCEH, ABCG1

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**Introduction**

In the past few decades, clinical research into type 2 diabetes and metabolic syndrome has revealed an interrelationship between the development of insulin resistance and atherosclerosis. The molecular mechanism of how insulin resistance and/or hyperinsulinemia induce atherosclerosis in type 2 diabetes has not yet been clarified.

Obesity is associated with enhanced generation of proinflammatory cytokines and lipids in adipose tissue. Signals by these mediators activate c-Jun NH2-terminal kinase (JNK) and an inhibitor of NF-κB, which leads to the generation of additional inflammatory mediators. These, in turn, lead to the suppression of insulin signaling through PI3-kinase, with the relative predominance of the other insulin-signaling pathway mediated by mitogen-associated protein kinase (MAPK). These events constitute insulin resistance and accompanying compensatory hyperinsulinemia which, in conjunction with the insulin signaling shifted to the MAPK pathway, predispose subjects to atherogenesis through vascular dysfunction and augmented mitogenic and prothrombotic events in the vascular wall.

Macrophages take up and store lipids to be transformed into atherosclerotic foam cells. As macrophages and foam cells do not degrade cholesterol, stored cholesteryl esters (CEs) must be hydrolyzed by hormone-sensitive lipase (HSL) and released free cholesterol (FC) is transferred to the plasma membrane. In a wide range of tissues, a single molecule of the
Materials and Methods

Cell Culture

THP-1 cells (Riken Cell Bank, Tokyo, Japan) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco) at 37 °C in 5% CO2. THP-1 cells were differentiated into macrophages by treatment with 200 nM phorbol 12-myristate 13-acetate (PMA; Biomol, Plymouth Meeting, PA, USA) for 72 h. For the measurement of cholesterol efflux, the cells were washed twice with PBS and incubated for 24 h in DMEM containing 10% FBS. The macrophages were incubated in the presence of HDL (50 μg/mL; Calbiochem (Darmstadt, Germany)) for 24 h and cholesterol efflux was determined by measuring radioactivity in the culture media and cells. Cholesterol efflux was calculated by dividing the radioactivity (dpm) in the culture media by the sum of the radioactivity in the medium and cells [(medium) / (medium + cells) × 100 (%)].

The effect of insulin on HDL-mediated cholesterol efflux was evaluated by adding different concentrations of insulin (0, 20, 100, or 500 nM; Wako Ltd., Kyoto, Japan) 30 min before the addition of HDL. These insulin concentrations were greater than the typical physiological concentration of insulin (from 1 to 10 nM) and in the range of diabetic hyperinsulinemia.

Quantitative Real-Time RT-PCR for nCEH, ABCA1 and ABCG1

Total RNA was extracted from macrophages using an RNeasy Mini Kit (QIAGEN, Japan). mRNAs for nCEH, ABCA1, ABCG1 and GAPDH were analyzed by real-time quantitative RT-PCR (ABI PRISM 7700 Sequence Detection System; Applied Biosystems, Foster City, CA, USA). The TaqMan probes for nCEH (HS00275607_m1), ABCA1 (HS0159122_m1), ABCG1 (HS00245154_m1) and GAPDH
Western Blotting for nCEH and ABCG1

Cells were lysed using Laemmli’s reducing sample buffer. The lysate was subjected to electrophoresis on a 6–9% gradient polyacrylamide gel, and the proteins were transferred to an Immobilon-P PVDF membrane (Millipore Corporation, Billerica, MA, USA). nCEH was detected using anti-carboxylesterase 1 rabbit IgG (1:5000; LifeSpan Biosciences, Seattle, WA, USA). Immunodetection was performed using HRP-labeled anti-rabbit IgG and a SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL, USA). ABCG1 protein was analyzed in a similar manner using a rabbit anti-ABCG1 antibody (1:200; Novus Biologicals, Littleton, CO, USA), and β-actin using an anti-actin rabbit antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Pictures of the membranes were downloaded to a computer and the intensity of each band was quantified with an NIH image (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Values are expressed as the mean ± standard deviation (S.D.). Differences among groups were determined using analysis of variance (ANOVA) with Bonferroni/Dunn post-hoc correction. Two groups were compared using the Mann-Whitney U test, and p < 0.05 was considered significant.

Results

Effect of Insulin on HDL-Mediated Cholesterol Efflux

Insulin inhibited, in a concentration-dependent manner, HDL-mediated cholesterol efflux from THP-1 macrophages (Fig. 1). Treatment with 500 nM insulin decreased cholesterol efflux by about 15% of the non-treated control: from 38.5 ± 2.2% to 31.6 ± 2.9% (n = 12).

Cholesterol Content in Macrophages

Cellular contents of TC and FC were decreased after incubation with HDL for efflux study. However, 100 or 500 nM insulin treatment disrupted this effect, as the reduction in cholesterol levels was significantly diminished compared with that in cells without insulin treatment (n = 6) (Fig. 2).

nCEH Activity and Expression in THP-1 Macrophages

The average values of nCEH activity in macrophages, determined before and after the efflux study, were 18.24 ± 7.36 and 24.94 ± 1.23 pmol/min/mg cell protein, respectively (n = 5) (Fig. 3). Treatment of the cells, 30 min prior to the efflux measurement, with insulin significantly suppressed nCEH activity after the efflux measurement, and the average activity values were 15.84 ± 3.19 pmol/min/mg cell protein with 100 nM insulin and 18.40 ± 0.82 pmol/min/mg cell protein with 500 nM insulin (n = 5).

Expressions of nCEH mRNA and protein are summarized in Fig. 4. There was a trend toward reduced nCEH mRNA levels after efflux; however, nCEH protein level increased by about 20%, which closely agreed with the result of enzyme activity. Pretreatment of the cells with insulin, at 100 or 500 nM, suppressed the expression of nCEH mRNA and protein.

ABCG1 Expression in THP-1 Macrophages

Expressions of ABCG1 mRNA and protein are summarized in Fig. 5. ABCG1 mRNA expression in the macrophages after efflux was reduced compared with the level before HDL-mediated cholesterol

(Hs99999905_m1) were Assays-on-Demand Gene Expression Products from Applied Biosystems.
Low levels of plasma HDL-cholesterol associated with hypertriglyceridemia and small-dense LDL are characteristic of diabetic patients with dyslipidemia. HDL plays a pivotal role in cholesterol efflux, which is the initial step of reverse cholesterol transport (RCT)\(^26\); however, the distinct mechanism of impaired RCT in type 2 diabetes is not well understood. Insulin has been reported to counteract cholesterol removal from human macrophages\(^23, 27\) and skin fibroblasts\(^28\), although the precise mechanism remains unclear.

Hydrolysis of CE to FC by nCEH is the initial process in the extracellular transport of cellular cholesterol. In the present study, insulin was demonstrated to affect cellular cholesterol efflux through the suppression of nCEH activity and ABCG1 expression. Several other factors, such as ACAT (acetyl-Coenzyme A acetyltransferase), ABCA1, SR-BI (scavenger receptor class B type I), CETP (cholesteryl ester transfer protein) and LCAT (lecithin-cholesterol acyltransferase), may also be involved in HDL-mediated cholesterol efflux\(^8-12, 14\). Regulation of these factors may be potentially related to the effect of insulin on cholesterol efflux.

Decreased intracellular cholesterol levels in macrophages ascertained the effect of HDL-mediated cholesterol efflux (Fig. 2). There were dissociations between the changes in mRNA and protein during efflux measurement for both nCEH and ABCG1: nCEH protein level was enhanced and ABCG1 protein remained unchanged despite reduced mRNA lev-
els. These results may suggest the presence of post-transcriptional regulation of nCEH and ABCG1 expressions. Similar post-transcriptional regulation has been reported in ABCA1 responding to ApoA-I.

In the present study, insulin reversed the decrease in cellular cholesterol content observed after HDL-mediated cholesterol efflux and was found to suppress the nCEH mRNA and protein levels of nCEH. *p<0.01

In adipocytes, lipolysis is regulated through the phosphorylation-mediated activation of HSL. HSL is phosphorylated by protein kinase A, which is activated with an increase in cyclic AMP levels in response to the stimulation of β-adrenergic receptors. Insulin counteracts β-adrenergic signaling through the activation of phosphodiesterase 3B, which, in turn, lowers cyclic AMP levels. Hydrolysis of CE to FC is executed by nCEH activity of HSL at least in macrophages.

Results of the present study suggest that insulin may exert an inhibitory effect on nCEH through the suppression of enzyme transcription, in addition to the phosphorylation-dependent regulation of enzyme activity.

The cell membrane transporters ABCA1 and ABCG1 are essential in cholesterol efflux, and the latter is highly expressed and plays an important role in efflux in macrophages. We found no significant changes in ABCA1 mRNA, the expression of which in macrophages is very low (data not shown).

Treatment with insulin further lowered ABCG1 mRNA and protein levels, which may be explained as the result of reduced cellular FC due to the inhibition of nCEH. The direct effect of insulin on ABCG1 expression has been addressed in several studies with inconsistent results, although several potential mechanisms have been suggested for insulin signaling.

Previous studies on hyperinsulinemia in human diabetics and in diabetic mice suggested the important role of elevated glucose in the down-regulation of ABCG1 in macrophages. As we performed the experiments with the same medium with a high glucose concentration (25 mM glucose in DMEM), the reduced levels of ABCG1 expression were not explained by the elevated levels of glucose.

Further studies are needed to elucidate the pre-

**Fig. 4.** Effect of insulin on nCEH expression in THP-1 macrophages.

Macrophages were pre-treated with insulin (100 or 500 nM) and exposed to HDL (50 µg/mL), and mRNA and protein for nCEH were analyzed by quantitative real-time RT-PCR (A) and Western blotting (B). Changes in nCEH mRNA and protein expressions closely agreed with those in enzyme activity. 100 or 500 nM insulin suppressed mRNA and protein levels of nCEH. *p<0.01

**Fig. 5.** Effect of insulin on ABCG1 expression in THP-1 macrophages.

Macrophages were pre-treated with insulin and exposed to HDL (50 µg/mL), and mRNA and protein for ABCG1 were analyzed by quantitative real-time RT-PCR (A) and Western blotting (B). Preincubation with insulin (100 or 500 nM) decreased mRNA and protein levels of mABCG1. *p<0.01
cise mechanism for the inhibitory effect of insulin on macrophage cholesterol efflux through ABCG1.

In conclusion, insulin reduces HDL-mediated cholesterol efflux from THP-1-derived macrophages through the inhibition of nCEH and ABCG1 expressions. Our findings provide further insight into the molecular mechanisms of the interrelationship between hyperinsulinemia and atherosclerosis, especially in insulin-resistant patients with type 2 diabetes and metabolic syndrome.

References
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