Human Resistin Stimulates Hepatic Overproduction of Atherogenic ApoB-Containing Lipoprotein Particles by Enhancing ApoB Stability and Impairing Intracellular Insulin Signaling

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Rationale: Obese individuals are at high risk for developing atherosclerosis primarily attributable to elevated plasma concentrations of apolipoprotein (apo)B-containing particles, including very-low-density lipoprotein (VLDL). Plasma levels of the adipose tissue adipokine resistin are increased in human obesity, and resistin expression is positively correlated with coronary atherosclerosis and VLDL levels.

Objective: We sought to determine for the first time whether resistin directly stimulates human hepatocyte production of apoB-containing particles and to elucidate the mechanisms responsible.

Methods and Results: Treatment of human hepatocytes with resistin at levels observed in human obesity stimulated apoB secretion up to 10-fold, because of increased microsomal triglyceride transfer protein (MTP) activity and decreased expression/phosphorylation of proteins in the insulin signaling pathways (insulin receptor substrate-2, Akt, and extracellular signal-regulated kinase). Resistin also increased hepatocyte lipid content by stimulating de novo lipogenesis via the SREBP1 and SREBP2 pathways. Furthermore, obese serum with elevated resistin levels induced greater hepatocyte stimulation of apoB secretion than lean human serum, an effect that was ameliorated by antibody immunoprecipitation removal of serum resistin.

Conclusions: Resistin has a direct deleterious impact on human hepatic lipid and lipoprotein regulation. Resistin greatly increased hepatocyte VLDL apoB and lipid secretion because of MTP activation and induction of hepatocyte insulin resistance. Conversely, antibody removal of serum resistin ameliorated human serum stimulation of apoB secretion. Increased hepatic cellular lipids mediated by resistin reflects the fatty liver/steatosis observed with elevated resistin in humans. Thus, human resistin is a novel therapeutic target for mitigating common hepatic pathophysiological processes associated with human obesity, dyslipidemia and atherosclerosis. (Circ Res. 2011;108:727-742.)

Key Words: obesity ■ dyslipidemia ■ apolipoprotein B ■ hepatic

The worldwide prevalence of obesity has reached epidemic proportions, with more than 1 billion individuals worldwide characterized as being obese. In North America alone, 1 in 3 adults is obese. This is a problem because obese individuals are at greatly elevated risk for developing atherosclerotic cardiovascular disease (ASCVD), the leading cause of death in North America.

Fundamental to the accelerated rate of ASCVD development in obese individuals is the presence of dyslipidemia. Despite numerous advances in the treatment of dyslipidemia, up to 60% of abdominally obese individuals have metabolic dyslipidemia: an elevation in plasma levels of triglycerides, a reduction in plasma high-density lipoprotein (HDL) cholesterol, and an increase in plasma numbers of low-density-lipoprotein (LDL) particles, which are small and dense. The primary lipoprotein abnormality that drives the development of metabolic dyslipidemia in obesity is an elevation in plasma levels of very-low-density-lipoprotein (VLDL), which precedes and is metabolically linked to each component of metabolic dyslipidemia.

Elevated VLDL in obesity is primarily attributable to increased hepatic secretion of VLDL triglycerides and apolipoprotein (apo)B. Although several mechanisms have been proposed, including whole-body and hepatic insulin resis-
levels, were not investigated. Conversely, another more recent study showed that whole-body gene deletion of resistin in mice that are either genetically obese (ob/ob) or induced to become obese through high-fat feeding results in significant reductions in plasma triglycerides and cholesterol and also reductions in in vivo VLDL-triglyceride secretion. Again, similar to the earlier study, the effects of physiological resistin levels were not investigated, neither were VLDL apoB metabolism or VLDL regulation at the hepatocyte level.

Translating the mouse data on the role of resistin in dyslipidemia development in obesity to humans is of tremendous potential importance in reducing the alarming ASCVD rates in human obesity. However, species-specific differences in resistin indicate that the results in mice may not necessarily translate to humans. There is only a 53% homology between the human and murine resistin genes. Moreover, some but not all prior studies have found significant associations between circulating resistin levels in humans and VLDL levels. Many of these studies, it should be noted, involved a small number of subjects. The more recent studies using more optimized resistin assays do show significant correlations between the two. A recent large population-based Framingham study, for example, did find highly significant associations between serum resistin levels and serum apoB and serum triglycerides. No study, however, has investigated a potential cause and effect relationship between resistin and VLDL levels in humans.

We therefore investigated the effects of human resistin on cellular VLDL regulation in human hepatocytes. We showed for the first time that human resistin directly mediates hepatic pathophysiological processes commonly associated with obesity. Resistin, at physiological concentrations, directly and potently stimulates atherogenic apoB/VLDL secretion and also results in the significant accumulation of hepatocyte lipids, indicative of hepatic steatosis. Mechanisms by which human resistin promotes hepatic apoB-containing lipoprotein oversecretion are multifold and include increased de novo lipogenesis via the SREBP1 and SREBP2 intracellular pathways, enhanced microsomal triglyceride transfer protein (MTP) activity, and reduced hepatic insulin signaling. These findings and the observation that antibody removal of human serum resistin ameliorates human serum stimulation of hepatocyte apoB secretion indicates that human resistin is an attractive and novel therapeutic target for mitigating hepatic dysregulation of lipid and lipoprotein processes, dyslipidemia, and ASCVD, commonly associated with obesity.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and includes detailed information regarding the following: cell culture protocols, real time RT-PCR, immunoprecipitation and Western blotting, oil red O for determination of cellular neutral lipid content, GC quantification of cellular lipids, EM photography of cell secreted lipoproteins, fast protein liquid chromatography (FPLC) lipoprotein distribution of secreted cell lipids, MTP enzyme activity assay, characteristics of patients used for human serum collection, ELISA for measurement of serum resistin, and statistical analysis.
Human Resistin Directly Stimulates Apolipoprotein ApoB Expression and Secretion in Human Hepatic HepG2 Cells

Cultured human hepatic HepG2 cells were maintained in 10% FBS-DMEM until 80% to 90% confluent, when HepG2 cells in 1% FBS-DMEM were treated with recombinant lyophilized purified human resistin (Calbiochem) reconstituted in millipore H2O. We confirmed that our human resistin source was indeed purified human resistin by applying the reconstituted human resistin to a 10% denaturing SDS-PAGE gel and using a rabbit polyclonal resistin antibody against human resistin (Santa Cruz) for detection. We observed a single 12 kDa band in our resulting immunoblot (Figure 1A), confirming that our resistin source was indeed purified human resistin.

All of the experiments to follow were performed three times and representative results are shown. We performed dose–response experiments in HepG2 cells, treating the cells with 0, 10, 25, 50 and 100 ng/mL of human resistin for 24 hours. We then performed immunoprecipitation on cell lysates and media, followed by Western blots to detect the cellular expression and secretion of apoB protein. The constitutively expressed cellular proteins, β-actin and albumin were used as internal immunoblot controls to confirm equal protein loading in our apoB immunoblots of cell lysates and media, respectively. Treatment with resistin at 10 ng/mL resulted in a 50% increase in hepatic apoB secretion versus control, untreated HepG2 cells (Figure 1B). Treatment with 25 ng/mL resistin, a plasma concentration of resistin reported in normal lean humans,14 further stimulated apoB secretion by 2-fold compared with untreated cells (Figure 1B).

Results

Human Resistin Directly Stimulates Apolipoprotein ApoB Expression and Secretion in Human Hepatic HepG2 Cells

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Figure 1. Physiological levels of human resistin stimulate human hepatocyte apoB100 protein in a dose–response manner. A, The human recombinant human resistin source used to stimulate human hepatocytes in all experiments was characterized via immunoblotting resistin. A total of 2 μg of the human resistin was immunoblotted using a 10% SDS gel and a polyclonal antibody against human resistin. The results confirmed the expected 12-kDa size of human resistin. B, Human resistin markedly stimulated the cellular expression and secretion of apoB protein in HepG2 cells (compared with control untreated cells) maintained in 1% FBS for 24 hours in a dose–response manner. C, The peak stimulatory effect of human resistin on HepG2 cellular expression and secretion of apoB protein was observed at a 50 ng/mL dose of human resistin applied to HepG2 cells for 24 hours. D, Human resistin (50 ng/mL, 24 hours) significantly stimulated the cellular expression of apoB protein in primary rodent hepatocytes isolated from fresh rat livers and in primary human hepatocytes from fresh human livers (both from Invitrogen), compared with control untreated hepatocytes, on 24 hours of treatment.
Remarkably, addition of 50 ng/mL human resistin to HepG2 cells, a concentration of resistin in the range typically reported for obese individuals, resulted in a 35-fold increase in cellular apoB protein expression and a 10-fold increase in secreted apoB versus untreated cells (Figure 1C). This finding demonstrates the highly potent effect of human resistin in mediating the production of apoB-containing lipoprotein particles and indicates a highly proatherogenic role for human resistin not previously identified.

Because the cellular expression of apoB protein was much greater than apoB secretion into the media with 50 ng/mL resistin, this indicates that much of the apoB synthesized as a result of resistin treatment is degraded before secretion. Nonetheless, the 10-fold elevation of apoB secretion induced by 50 ng/mL resistin treatment above control, untreated cellular levels, would be expected to greatly deteriorate the dyslipidemic profile of obese individuals because the plasma levels of apoB-containing lipoproteins in humans are largely determined by their hepatic rates of production. This is the key finding of this study.

Although a clear dose-response positive relationship was seen between 0 to 50 ng/mL human resistin on HepG2 apoB protein secretion, treatment of HepG2 cells with 100 ng/mL resistin, surprisingly reduced cellular apoB expression markedly and reduced its secretion versus that produced by 50 ng/mL resistin (Figure 1C). This amount of resistin (100 ng/mL) is a supraphysiological amount of resistin and we can surmise that it produced a negative-feedback effect on apoB protein production.

Human Resistin Directly Stimulates ApoB Expression in Primary Human and Rodent Hepatic Cells

To determine whether the stimulatory effect of human resistin on hepatocyte apoB protein expression in the above in vitro findings is relevant to humans and to rodent models, we treated mouse and rat hepatocytes freshly isolated from livers and cryogenically preserved human hepatocytes isolated from fresh human livers with human resistin at the optimum 50 ng/mL dose for 24 hours. Note that the recommended media for the primary hepatocytes was not optimal for maintaining the stability of apoB protein secreted into the media and we were thus not able to identify robust apoB protein bands from media. However, cellular apoB100 protein expression was clearly visible from primary cell lysates. As with our HepG2 cell results above, we observed significantly greater apoB protein expression in human resistin-treated cells versus untreated control cells in all species tested (mouse, rat and human) (Figure 1D). The percentage increases seen were in the 40% to 50% range for all three species. Although the magnitude of the stimulatory effect of human resistin on cellular apoB protein expression in primary hepatocytes was not as large as with HepG2 cells, this was expected because primary hepatocytes are not as metabolically active as cultured hepatoma HepG2 hepatocytes. Nonetheless, we did confirm, importantly, that our key finding is applicable to humans and rodent models.

Human Resistin Stimulation of Hepatic ApoB Expression and Secretion Is Prolonged, Rapid, More Potent Than Oleate, Occurs in Both Normal and Fat-Accumulated Hepatocytes, and Does Not Induce Cellular Apoptosis in HepG2 Cells

We wished to determine whether the acute stimulatory effect of human resistin on hepatic apoB protein expression and secretion is maintained for a prolonged period of time. We observed that the stimulatory effect of 50 ng/mL resistin treatment (the dose of resistin that we found to be most deleterious on hepatocyte apoB secretion) on HepG2 apoB secretion observed at 24 hours was maintained after 48 hours (not shown), demonstrating the prolonged stimulatory effect of human resistin on hepatic apoB. This can be interpreted in at least 2 ways. One interpretation is that the resistin peptide is very stable in cellular media; another possibility is that resistin induces a prolonged enhancement of the cellular machinery that stimulates apoB production and/or stability.

We wished to further investigate the time-course effects of human resistin on hepatic apoB secretion. A steady increase in apoB protein secretion into HepG2 cell media was observed as early as 4 hours after resistin treatment and up until the 24-hour length of the experiment, when the maximal cumulative effect on apoB protein expression in the media was observed (Figure 2A). Because apoB transcription in human hepatocytes has been reported to require 16 hours and apoB translation requires less than 15 minutes, this indicates that cellular mechanisms which are intermediate to apoB mRNA and protein synthesis processes are enhanced by human resistin. Stimulation of hepatic cellular enzymes which enhance apoB stability, decreased expression of key proteins in the intracellular insulin signaling pathway, and increased cellular neutral lipid content, all of which are known to enhance apoB stability, are plausible mechanisms that we investigated (described in a further section of the Results below).

Next, to determine the magnitude or potency of the effect of human resistin on hepatic apoB expression and secretion, we compared the effect of the abundant plasma monounsaturated FFA, oleate, traditionally and commonly used to stimulate hepatic apoB production, to that of resistin. This is particularly of concern in obese humans, in whom plasma FFA levels, including that of oleate, are elevated. Exposure of hepatocytes to 100 μmol/L oleate for 24 hours increased cellular apoB expression and secretion by 55%, in comparison with the 10-fold increase in apoB secretion induced by 50 ng/mL described above (Figure 2B). To give a clearer idea of the potency of human resistin to oleate, 50 ng/mL of resistin is equivalent to 4.6 pmol/L or 4.6 × 10^{-6} μmol/L resistin, in comparison with the 100 μmol/L oleate that we used to stimulate the human hepatocytes. Thus, human resistin is potentially a highly potent therapeutic human drug target. Addition of 100 μmol/L oleate to resistin (50 ng/mL, 24 hours) did not further stimulate hepatocyte apoB expression or secretion.

The next question was whether this amount of human resistin is toxic to hepatocytes. We therefore performed trypan blue staining of HepG2 cells on 50 ng/mL of resistin treatment to determine cell viability. The results showed that no reduction in hepatocyte viability was seen with either 50 ng/mL or 100 ng/mL human resistin treatment for 24 hours (not shown).
The elevated plasma FFA levels observed frequently in obese humans, as discussed above, contributes in large part to the pathogenic ectopic fat distribution and fatty livers commonly observed in these individuals. Therefore, a key further question is whether a model of “fatty liver” transformed hepatocytes would react differently to human resistin than normal hepatocytes. To test this question, we preloaded HepG2 cells with 500 μmol/L oleate for 24 hours before 24-hour resistin stimulation of the cells. This amount of oleate was sufficient to increase neutral lipid content of the cells, without inducing cell toxicity. Our results showed that although resistin still significantly increased hepatocyte apoB secretion in oleate preloaded cells, the magnitude of the apoB-stimulatory effect of resistin was diminished (≈55% above control, untreated cells) (Figure 2C). Such a decrease in apoB secretion with high and prolonged FFA incubation of hepatocytes has been reported before and may be a protective effect of the liver to avoid very large increases in hepatic VLDL production. Alternatively, it may contribute to liver pathogenesis such that in conditions of high peripheral FFA concentrations and elevated FFA accumulation in the liver, the liver FFA is shunted to its neutral, storage lipid form (triglycerides), which then further contributes to and worsens the fatty liver phenotype.

**Figure 2. Time course effects of human resistin and comparative effects to oleate on human hepatocyte apoB100 protein.**

A. Time course study of human resistin-treated HepG2 cells (50 ng/mL) showed a significant increase in apoB secretion as early as 4 hours after treatment vs time 0, and cumulative apoB secretion continuing on to the 24-hour time frame of the study. B. Stimulatory effect of human resistin (50 ng/mL) on HepG2 apoB100 cellular expression and secretion (24 hours) is much more potent than the FFA, oleate (100 μmol/L). C. Pre-loading HepG2 cells with oleate (500 μmol/L) to reflect cellular fatty acid accumulation in a model of fatty liver, diminished the effect of resistin (50 ng/mL, 24 hours) on apoB.

**Human Resistin Stimulates the Secretion of Atherogenic VLDL Particles in HepG2 Cells**

We next investigated the characteristics of the apoB-containing lipoprotein particles produced as a result of treatment of human hepatocytes with human resistin. FPLC (size exclusion chromatography) was used to determine the effect of human resistin treatment on the lipid composition of lipoproteins secreted by HepG2 cells. We compared the lipoprotein distribution of secreted lipids by HepG2 cells after 24 hour incubation with human resistin at a concentration of 50 ng/mL, the most effective resistin dose, and compared it to untreated control HepG2 cells. We collected concentrated media from 6- to 10-cm plates from each treatment group. The concentrated media was
then injected into Superdex FPLC columns, fractionated and eluted and individual fractions were analyzed for triglycerides and cholesterol. The results showed large increases in the triglyceride and cholesterol contents of the secreted VLDL fraction as a result of human resistin treatment, with virtually no change in lipid contents of the secreted LDL or HDL fractions. There was no difference in the secreted glycerol peaks or profiles between control, untreated cells and resistin-treated cells. The magnitude of the increase in secreted VLDL triglycerides and cholesterol was more than 8- to 9-fold with human resistin treatment, relative to control untreated cells (Figure 3A and 3B).

As indicated in the first section of the Results above, the secretion of apoB protein by HepG2 cells increased markedly by approximately 10-fold with human resistin treatment, along with the resistin-mediated increase in secreted VLDL lipids described here. Thus, increased secreted VLDL lipid components by HepG2 cells treated with resistin occurred concurrently with an increase in secreted VLDL particle numbers with resistin. Therefore, with resistin treatment, individual VLDL particles would be expected to have smaller content of triglyceride and cholesterol compared to VLDL particles secreted by control, untreated HepG2 cells. This would indicate that the type of VLDL particles secreted by resistin-treated hepatocytes are smaller, more numerous, and therefore more atherogenic than that secreted by control, untreated hepatocytes.\textsuperscript{28,29} Small, dense apoB-containing particles have been reported to more readily enter the arterial intima, thereby initiating and accelerating atherosclerotic plaque formation in arterial walls.\textsuperscript{28,29}
To confirm whether this is the case, we performed electron microscopy (EM) analyses on media from HepG2 cells, either untreated or treated with 50 ng/mL resistin for 24 hours. The VLDL fraction from the media was first isolated via density ultracentrifugation of the media at d1.006 g/mL. The VLDL fraction was then fixed and stained with osmium and imaged using EM. EM analysis using the NIH Image J software program showed a mean VLDL diameter of 80 nm in the resistin-treated samples, which was less than that of control, untreated samples (Figure 3C). Furthermore, the quantity of VLDL particles in a representative 100 cm² area was greater in resistin-treated samples by a mean of 10-fold (Figure 3C). Note that these results are derived from HepG2 cells and should in future also be repeated in an in vivo setting on resistin treatment in animal model and human settings.

We also characterized the apoC and apoE protein expression by HepG2 cells treated with human resistin (50 ng/mL) and compared them to that secreted by control, untreated HepG2 cells. There was no difference in apoCI or apoCIII protein expression in HepG2 cells treated with or without resistin (not shown). This suggests that the VLDL particles secreted by hepatocytes treated with human resistin are not different in terms of their capacity to activate lipoprotein lipase mediated lipid hydrolysis or lipolysis, a major circulatory remodeling enzyme. There was, however, an increase in the expression of secreted apoE in human resistin-treated hepatocytes (Figure 3D), which is a ligand for hepatic lipase, which does induce lipoprotein lipolysis at hepatocytes and can induce the formation of smaller, denser lipoprotein particles.

### Human Resistin Induces Hepatic Lipid Accumulation in HepG2 Cells

We wished to determine whether the deleterious hepatic effects of human resistin extend not only to increased secretion of apoB-containing lipoproteins, but also to increased hepatocyte lipid content, as the 2 processes frequently occur together in such conditions as obesity, insulin resistance and type 2 diabetes mellitus.30,31 We therefore performed oil red O/hematoxylin staining of HepG2 cells either untreated, treated with 100 μmol/L oleate for 24 hours as a positive control, or treated with 50 ng/mL or 100 ng/mL human resistin for 24 hours. The experiments using resistin were performed in the absence of oleate to assess the direct effect of human resistin on hepatocyte neutral lipid accumulation, independent of the effect of exogenous fatty acids, and also, to compare the effect of resistin with that of oleate. The results showed a clear increase in hepatocyte neutral lipid content in human resistin-treated HepG2 cells versus control, untreated HepG2 cells (Figure 4A). The increased cellular neutral lipid content with resistin treatment was similar in magnitude to that observed with 100 μmol/L oleate treatment.

To quantify the hepatocyte lipid changes induced by human resistin, we performed gas chromatography (GC) analysis of lipids extracted from harvested hepatocytes either untreated or treated with human resistin (50 ng/mL) for 24 hours. GC analyses showed a 25% increase in triglyceride content, an 15% increase in cholesteryl ester content, and a 3% increase in the free cholesterol content in hepatocytes treated with resistin for 24 hours (Figure 4B). Therefore, the results indicate that human resistin acts acutely to markedly increase hepatocyte triglycerides and cholesterol and can potentially directly induce fatty liver and hepatic steatosis concomitant with increasing hepatic VLDL secretion.

### Human Resistin Stimulation of ApoB Is Partly Mediated at the mRNA Level

Results from our real-time RT-PCR analyses demonstrated that human resistin stimulation of hepatocyte apoB expression is partly mediated at the transcriptional level. There was a significant increase in apoB mRNA expression in HepG2 cells with 50 ng/mL human resistin treatment for 24 hours (Figure 5A). In contrast, apoB mRNA levels were at control levels with 100 μmol/L oleate treatment.

### Key Mechanisms By Which Human Resistin Stimulates Hepatocyte ApoB Expression and Secretion: Increased MTP Activity and Reduced Insulin Signaling

We wished to investigate mechanisms by which human resistin stimulated increased hepatocyte expression and secretion of apoB protein. Because our time-course hepatocyte apoB secretion study described above showed a much more rapid increase in apoB secretion than would be required for increased apoB transcription solely, and because it is well known that apoB is primarily regulated by co- and post-translational mechanisms, particularly co- and post-translational apoB degradation, we sought to investigate regulators of cellular apoB degradation.3 We first determined whether human resistin inhibits apoB degradation through the classic proteasome-dependent degradation pathway by adding the optimal dose of human resistin (50 ng/mL) to the proteasome-dependent inhibitor of apoB degradation, lactacystin, at the optimal dose and time (10 μmol/L, 24 hours).32 We found a similar increase in the cellular expression and secretion of apoB in HepG2 cells with lactacystin and resistin, indicating that human resistin acts to increase hepatocyte apoB primarily through inhibition of proteasome-mediated apoB degradation.

Increased hepatocyte availability of lipids for incorporation with apoB is a key mechanism by which apoB degradation is inhibited3 and indeed, as indicated in the Results section above, human resistin increased cellular neutral lipids, which can then be accessed by apoB during its assembly into VLDL particles. The intracellular endoplasmic reticulum (ER) apoB chaperone and enzyme, MTP, is crucial for the transfer of such lipids to apoB and is thereby a key regulator of intracellular apoB degradation/stability.8 Assessment of cellular MTP protein expression and lipid transfer activity, in HepG2 cells in response to human resistin treatment (50 ng/mL, 24 hours) showed significant increases in both MTP parameters, demonstrating for the first time, that human resistin directly stimulates MTP in human hepatocytes (Fig-
MTP mRNA levels, however, were not altered by resistin. Another important regulator of hepatocyte apoB/VLDL assembly and degradation/stability is the intracellular insulin signaling pathway. Reduced signaling activity in this pathway has been shown to enhance apoB stability, both directly and indirectly, by enhancing the cellular availability of lipids, and also by inducing increased MTP expression. We found that human resistin (50 ng/mL, 24 hours) significantly decreased the expression of key hepatocyte proteins in 2 key intracellular insulin signaling pathways by 15% to 50% (Figure 5C): insulin receptor substrate (IRS)-2, Akt, and phosphorylated Akt(Ser473) in the Akt/PI3 kinase (Akt/PI3K) pathway and extracellular signal-regulated kinase (ERK) and phosphorylated ERK in the mitogen-activated protein kinase/ERK (MAPK/ERK) pathway. Although resistin-mediated decreases in IRS-2 and serine phosphorylated Akt have previously been reported in human hepatocytes, our results showing human resistin reduction of insulin signaling in the MAPK/ERK pathway is a novel finding.

Figure 4. Examination of the lipid content of HepG2 cells stimulated with human resistin vs control hepatocytes. Cells were either untreated (control hepatocytes) or treated with: 100 μmol/L oleate, 50 ng/mL resistin (in the absence of oleate), or 100 ng/mL resistin (in the absence of oleate). A, Oil red O/hematoxylin staining of neutral lipids in HepG2 cells showed a greater cellular neutral lipid content with 50 ng/mL resistin treatment vs untreated control cells, which was comparable to 100 μmol/L oleate treatment, but not to 100 ng/mL resistin treatment, after 24 hours. B, Gas chromatography (GC) analyses of HepG2 cell extracted lipids after 24 hours of treatment with 50 ng/mL human resistin showed significant increases in cellular triglycerides and cholesteryl esters, with lesser increases in cellular free cholesterol content.

Increase in Cellular Neutral Lipid Content and VLDL Lipid Secretion Induced by Human Resistin Is Mediated Through the Induction of the Cellular SREBP1 and SREBP2 Lipogenic Pathways but Not by Changes in AMP Kinase

We wished to determine whether the resistin-mediated increase in hepatocyte lipid content and secreted VLDL lipids is attributable to increased cellular de novo lipogenesis. As intracellular neutral lipids inhibit intracellular apoB protein degradation and enhance apoB protein stability, the increase in cellular neutral lipids observed with resistin treatment explains in part the enhanced apoB protein expression and subsequent increase in apoB protein secretion with resistin. Indeed, the results showed significantly increased mRNA expression of SREBP1 and SREBP2 genes, the master transcription factors in the fatty acid/triglyceride and cholesterol cellular biosynthesis pathways, respectively, on HepG2 human resistin treatment (50 ng/mL, 24 hours) (Figure 6A). This was associated with a significant 3-fold increase in the expression of ACC (acylcoenzyme A carboxylase) and significant 2-fold increases in the mRNA expression of key SREBP2 intracellular cholesterol biosynthetic target enzymes: HMG-CoA reductase, HMG-CoA synthase, and squalene synthase (Figure 6B). In terms of the SREBP1 pathway, SCD, which mediates intracellular monounsaturated fatty acid biosynthesis, and DGAT1, which mediates intracellular triglyceride biosynthesis, increased by smaller but significant extents with hepatocyte human resistin treatment (Figure 6C).

Another potential and well-described mechanism by which hepatocyte cellular lipid content can be increased is via decreased lipid/fatty acid oxidation via the AMP kinase (AMPK) pathway. We therefore determined the effect of human resistin (50 ng/mL, 24 hours) on HepG2 AMPK and phosphorylated (p)AMPK protein expression via Western blot. We found no change in the expression of either AMPK
proteins with resistin treatment. A previous study did report a small, significant decrease in pAMPK protein expression with human resistin treatment of HepG2 cells, a difference that may be attributed to antibody differences in detection of AMPK subunits (the α2 subunit in the prior study and both α1 and α2 subunits in the present study) and phosphorylation sites of pAMPK (Ser473 in the prior study and Thr172 in the present study).  

Figure 5. Mechanisms accounting for stimulation of apoB protein expression and secretion by human resistin treatment (50 ng/mL, 24 hours) of HepG2 cells. A, Resistin treatment resulted in a significant increase in cellular apoB mRNA content vs untreated control cells, as assessed by real-time RT-PCR. B, Resistin significantly increased both cellular MTP protein expression and MTP activity. C, Resistin significantly decreased hepatocyte expression of key proteins in the intracellular insulin signaling pathway, including IRS-2, ERK, phosphorylated ERK, Akt, and phosphorylated Akt. D, Resistin treatment did not significantly alter hepatocyte expression of AMPK or its active, phosphorylated form.
Removal of Resistin in Human Serum Ameliorates the Stimulatory Effect of Obese Human Serum on Hepatic ApoB Secretion

We wished to examine whether, in a physiologically relevant human setting, resistin stimulates hepatocyte apoB secretion. We therefore incubated HepG2 cells with serum (10% in DMEM for 24 hours) from metabolically well characterized obese (19 individuals with BMI >30 kg/m² and <35 kg/m²) and lean (17 individuals with BMI ≤25 kg/m²) humans from the multiethnic M-CHAT (Multicultural Community Health Assessment Trial) study.

For the present study, serum was obtained solely from male patients from the M-CHAT study (N=36). Patients used in the present study had a mean age of 50 years (Online Table I). Lean individuals had a mean BMI of 23 kg/m², a mean waist circumference (WC) of 84 cm, whereas obese individuals had a mean BMI of 32 kg/m² and a mean waist circumference of 106 cm (Online Table I). Subjects from both European white and South Asian ancestry were included. Lean and obese subjects had similar serum total cholesterol and LDL-cholesterol levels and similar glucose levels (Online Table I). As expected, obese subjects had significantly greater serum triglyceride and lower HDL-cholesterol concentrations than their lean counterparts (Online Table I).

We performed 24-hour incubation of HepG2 cells with 10% serum from our well-characterized human lean and obese (19 individuals with BMI >30 kg/m² and <35 kg/m²) humans from the M-CHAT study (N=36). Patients used in the present study had a mean age of 50 years (Online Table I). Lean individuals had a mean BMI of 23 kg/m², a mean waist circumference (WC) of 84 cm, whereas obese individuals had a mean BMI of 32 kg/m² and a mean waist circumference of 106 cm (Online Table I). Subjects from both European white and South Asian ancestry were included. Lean and obese subjects had similar serum total cholesterol and LDL-cholesterol levels and similar glucose levels (Online Table I). As expected, obese subjects had significantly greater serum triglyceride and lower HDL-cholesterol concentrations than their lean counterparts (Online Table I).

We performed 24-hour incubation of HepG2 cells with 10% serum from our well-characterized human lean and obese individuals. The results demonstrated a striking and significant 5- to 8-fold greater stimulatory effect of obese human serum on cellular apoB protein expression versus lean controls (determined via immunoprecipitation and Western blot of cell lysates (Figure 7A). This is the first identification of stimulatory effect of obese human serum on hepatocyte apoB secretion. Serum resistin levels were further measured in all subjects via ELISA, showing a significant 50% elevation in serum resistin levels in obese versus lean individuals, associated with the greater obese serum stimulation of hepatocyte apoB, and implicating elevated serum resistin in obesity with increased hepatocyte apoB production (Figure 7B).

We also compared the effects of lean serum stimulation of hepatocyte cellular apoB expression (24 hours) with serum-free incubation of hepatocytes and found, surprisingly, that apoB expression was 30% lower with lean serum stimulation of hepatocytes versus serum-free controls (Figure 7C). This inhibitory effect of lean serum on hepatocyte apoB expression should be further investigation in future studies.

To further determine whether resistin in human serum directly plays a quantitatively important role in mediating hepatocyte apoB production, we performed immunoprecipitation polyclonal antibody removal of serum resistin, and examined the subsequent effect on cellular apoB expression. Antibody removal of resistin in lean human serum diminished cellular apoB significantly and remarkably by 50%; antibody removal of resistin in obese serum significantly reduced cellular apoB by 30% (Figure 7D). These results indicate that resistin in human serum plays a quantitatively important role in mediating hepatocyte apoB production. This further indicates that reduction or inhibition of serum resistin in humans is a potentially effective treatment for hepatic VLDL overproduction and dyslipidemia, both in obese and nonobese states.

Discussion

Here, we have showed for the first time that human resistin directly and potently stimulates VLDL apoB protein and lipid secretion by human hepatocytes. This key finding expands our understanding of resistin pathophysiology in humans and extends the recent reports of resistin-induced dyslipidemia in rodents. This is notable because the strong rodent data on the pathophysiological effects of resistin in mediating other metabolic impairments, including insulin resistance and inflammation, have not necessarily been translated to humans.

Despite the gap in our understanding of the role of resistin in humans, circulating resistin levels are increased in human obesity and have been shown to be correlated to BMI and visceral body fat in recent studies in humans. Therefore, a role or roles for resistin in mediating metabolic impairments in human obesity have been proposed. This is more so because plasma resistin levels in humans are predictive of cardiovascular diseases (CVDs), such as coronary atherosclerosis, independent of the presence of obesity.
The finding in the present study that human resistin directly and potently stimulates VLDL apoB and lipid secretion in humans may explain, at least partly, the positive association of resistin with CVD in humans, although this needs to be specifically tested in a clinical setting. This is because elevated plasma VLDL levels are a driver for dyslipidemia development (in particular, metabolic dyslipidemia), the presence of which has been found in prior studies to be among the strongest risk factors for accelerated CVD development in humans.6,41

In the present study, we found a positive dose–response effect of human resistin (ranging from 0 to 50 ng/mL) in stimulating hepatocyte apoB protein expression and secretion. The stimulatory effect of resistin on apoB was present at all doses, to a maximum at 50 ng/mL resistin, a serum level of resistin reported in obese human subjects.14 At this dose, resistin produced a nearly 10-fold increase in apoB protein secretion into hepatocyte media versus control, untreated cultured hepatocytes. Both the cellular expression and secretion of apoB were upregulated with resistin, indicating that cellular processes involving the synthesis and/or stability of apoB protein (both of which we confirmed) were stimulated by resistin, and that assembly of apoB into lipoprotein particles destined for secretion was enhanced. Our finding above in HepG2 cells that human resistin enhances hepatocyte apoB protein expression were confirmed in primary hepatocytes isolated from mouse, rat, and human livers, demonstrating the in vivo relevance of our findings.

This enhancement occurred independent of any marked increase in external sources of FFAs, a substrate and stimulator of hepatocyte VLDL production. This finding is consistent with some, but not all, previously published studies that concluded that although plasma FFA levels are frequently increased in obese humans with elevated plasma VLDL concentrations, an increase in peripheral FFA is not necessary...
for elevating hepatic VLDL production in humans. Our findings, conversely, are consistent with the mice data demonstrating that resistin gene deletion or overexpression alters in vivo VLDL production despite lack of changes in serum FFA levels.

We further demonstrated that resistin is a much greater stimulator of hepatocyte apoB than FFA. We exposed hepatocytes to 100 μmol/L olate, a FFA traditionally used to stimulate hepatic apoB production, and compared them to hepatocytes treated with 50 ng/mL resistin, with and without the addition of olate. The apoB stimulatory effect of resistin was much more potent than that of olate, because the amount of resistin used in this experiment (50 ng/mL) is the equivalent of 4.6×10⁻⁶ μmol/L resistin, compared with the 100 μmol/L olate used. This suggests that resistin is potentially a highly potent therapeutic target in humans.

The elevated plasma FFA levels observed frequently in obese humans, as discussed above, contribute in large part to the pathogenic ectopic fat distribution and fatty livers commonly observed in these individuals. Therefore, a key further question was whether a model of “fatty liver” transformed hepatocytes would react differently to human resistin than normal hepatocytes. To test this question, we preloaded HepG2 cells with 500 μmol/L olate for 24 hours before resistin stimulation (50 ng/mL, 24 hours) of the cells. This amount of olate was sufficient to increase the neutral lipid content of the cells, without inducing cell toxicity. Our results showed that although resistin still significantly increased hepatocyte apoB secretion in olate preloaded cells, the magnitude of the apoB-stimulatory effect of resistin was diminished (approximately 55% above control, untreated cells). Such a decrease in apoB secretion with high and prolonged FFA incubation of hepatocytes has been reported before and may be a protective effect of the liver to avoid very large increases in hepatic VLDL production. Alternatively, it may contribute to liver pathogenesis such that in conditions of high peripheral FFA concentrations and elevated fatty acid accumulation in the liver, liver fatty acids are shunted to their neutral, storage lipid form (triglycerides), which then further contributes to and worsens the fatty liver phenotype.

An advantage to our present studies in human hepatocytes, compared to the whole-body mice studies investigating the role of resistin in dyslipidemia and VLDL generation, is that we assessed the direct hepatic effects of resistin, independent of possible cross-talk from signaling molecules derived from adipose tissue to the liver. Resistin is well known to affect the expression and secretion of other adipokines such as adiponectin and leptin and cytokines such as TNF-α, which on their own can have effects on hepatic lipoprotein regulation via adipose-hepatic intertissue communication.

Moreover, in the present study, we determined the impact of resistin on hepatic VLDL generation without the confounding effects of chronic hyperinsulinemia present in the mouse studies. The studies carried out thus far on resistin-mediated dyslipidemia development in animals have been limited to mice with adenoviral overexpression of resistin or whole-body deletion of the resistin gene, both of which resulted in chronically altered circulating insulin levels, and, thus, the independent effects of resistin without hyperinsulinemia could not be determined. Hyperinsulinemia is seen frequently in many but not all obese individuals. Chronic hyperinsulinemia and insulin resistance have been showed to be partially responsible for elevated VLDL production in several in vivo and cell culture models.

Previously, the direct effect of resistin in inhibiting insulin signaling in the PI3 kinase/Akt pathway was demonstrated in human hepatocytes and, indeed, here, in our studies, we identified a role for resistin-mediated hepatocyte insulin resistance playing a role in the apoB stimulatory effect of resistin. More specifically, we confirmed significantly reduced expression of key proteins in the PI3 kinase/Akt insulin signaling pathway (IRS-2, Akt and phosphorylated Akt), and, for the first time, showed reduced expression of proteins in the MAPK/ERK insulin signaling pathway (ERK and pERK), as a direct result of human resistin treatment.

Whereas, in our dose–response resistin studies described above, we did demonstrate a dose–response increase in hepatocyte apoB protein expression and secretion with increasing physiological quantities of human resistin, we surprisingly found a sharp decline in hepatocyte apoB cellular expression and secretion with 100 ng/mL of resistin, a supraphysiological quantity of resistin, which has not been reported in humans. Because we did not observe any decrease in hepatocyte viability with the supraphysiological resistin dose, increased cellular apoptosis likely does not explain the decline apoB protein expression and secretion with this dose of resistin. The possible cause of this reversal in apoB protein levels may be a negative-feedback effect of this quantity of resistin on the proteins directly involved in apoB production and stability (eg, MTP) or the signaling pathways that enhance apoB stability (eg, the lipogenic and insulin signaling pathways). It may also be attributable to invagination and inactivity of the putative resistin receptors on supersaturation by supraphysiological amounts of resistin. This process has been described before for other serum factors. Consistent with this notion, saturation of the putative resistin receptor, and plateau in resistin effects, have previously been described in rodent studies investigating the metabolic effects of resistin. These possibilities will require investigation in a human setting in future studies.

In addition to the dose–response effects of resistin, we assessed the time course of the effects of resistin on apoB secretion and found that, at the optimal resistin dose (50 ng/mL), apoB secretion steadily increases in a curvilinear fashion with time, to a peak at 24 hours. An increase in apoB secretion was seen as early as 4 hours; because the length of time required for increased transcription of apoB is 16 hours, processes involving apoB translation and/or stability, which require less time, must also be involved. Notably, the stimulatory effect of resistin was still seen at 48 hours, indicating prolonged apoB protein stimulation by resistin.
We did observe an increase in apoB mRNA levels with resistin, an effect not previously observed with other traditional apoB stimulators, such as olate and insulin, which has, therefore, lead many investigators to conclude that apoB is principally regulated at the translational and post-transcriptional levels. Here, we demonstrated transcriptional control of apoB with human resistin.

In addition to resistin-mediated stimulation of apoB transcription, processes involved in maintaining apoB stability/decreasing apoB degradation (that is, post-transcriptional control of apoB) were also enhanced by resistin. We first determined whether human resistin inhibits apoB degradation through the classic proteasome-dependent degradation pathway by adding human resistin (50 ng/mL) to the proteasome-dependent inhibitor of apoB degradation, lactacystin. We found similar increases in the cellular expression and secretion of apoB in HepG2 cells with lactacystin and with resistin, indicating that human resistin acts to increase hepatocyte apoB primarily through inhibition of proteasome-mediated apoB degradation.

The intracellular endoplasmic reticulum (ER) apoB chaperone and enzyme MTP is crucial for the transfer of lipids to apoB and is thereby a key regulator of intracellular apoB stability/degradation. Assessment of cellular MTP protein expression and lipid-transfer activity (that is, triglyceride transfer activity) in HepG2 cells in response to human resistin treatment (50 ng/mL, 24 hours) showed significant increases in both MTP parameters, demonstrating, for the first time, that human resistin directly stimulates MTP in human hepatocytes. The resistin-mediated stimulatory effect on MTP activity occurred rapidly, within 24 hours, whereas the reported half-life of MTP is 4 days. Such a rapid effect on MTP activity by resistin can be explained by its stimulatory effect on de novo MTP protein synthesis.

Our observed resistin-mediated increase in apoB protein secretion by human hepatocytes indicates that resistin increases secreted VLDL particle numbers, because each VLDL particle secreted contains one apoB molecule. We confirmed this finding with EM photography of the VLDL particles in the resistin-treated hepatocyte media, which showed an approximately 10-fold increase in particles of the VLDL size range versus control-untreated hepatocyte media.

Consistent with the resistin-mediated increase in secreted VLDL apoB protein by human hepatocytes, there was a more than 8- to 9-fold increase in secreted lipids in the VLDL fraction, assessed using FPLC size-exclusion separation of hepatocyte media. Both VLDL triglycerides and cholesterol were increased as a result of resistin (50 ng/mL) treatment. Control, untreated HepG2 hepatocyte media (with no FBS added) showed virtually no particles (but not none) in the VLDL size range, which is consistent with previous studies showing this anomaly in this human cell line. In contrast to the large resistin-mediated increases in secreted VLDL lipids by hepatocytes, there was minimal change in secreted LDL or HDL lipids, indicating a specific, exclusive and directed effect of human resistin on VLDL secretion.

In addition to the composition of VLDL lipids and protein, the size of VLDL particles secreted in response to resistin treatment was determined via EM photography, and were shown to be smaller than VLDL particles produced by control, untreated human hepatocytes. This may be attributable either to resistin inducing smaller nascent VLDL particles to be secreted by hepatocytes or to resistin stimulating greater lipolysis of the nascent secreted VLDL particles by cell surface lipases, which cause smaller, denser particles to be generated. The main lipase on the surface of hepatocytes is hepatic lipase. Because we found an increase in apoE protein secretion in resistin-treated hepatocytes, and apoE is a known hepatic lipase ligand, the increased apoE may have enhanced the VLDL lipolysis process in resistin-treated cells. In contrast, levels of secreted apoC (apoCII or CIII) were not altered by resistin. ApoCII and -CIII are known to alter the activity level of the other dominant cell surface lipase in humans: the extrahepatic lipase lipoprotein lipase. Because there was no change in secreted levels of apoC, we would not expect any change in lipoprotein lipase-mediated lipolysis of the VLDL particles produced by resistin, once they entered the circulation. All in all, because VLDL particle numbers were markedly increased and VLDL size was decreased by human resistin, this indicates that greater numbers of smaller, more atherogenic VLDL particles that can more readily enter arterial walls and induce atherosclerosis are generated by resistin. Notably, this may be the reason why elevated serum resistin levels in humans predict increased coronary atherosclerosis.

Along with the increase in secreted VLDL lipids stimulated by hepatocyte resistin treatment, there was a marked increase in cellular neutral lipid content, visualized by oil red O staining. The magnitude of the resistin-mediated increase in hepatocyte lipids was similar to that of olate-treated hepatocytes. This shows a potentially pathogenic steatotic effect of human resistin and is consistent with the increased incidence and association of hepatic steatosis in humans with elevated serum resistin levels. We further quantified the specific lipids altered in hepatocytes as a result of resistin treatment and showed, through GC quantification, that hepatocyte triglycerides increased markedly by 25%, cholesterol esters by 15% and free cholesterol by 3%, versus control, untreated hepatocytes.

The dual increase in secreted VLDL lipids and hepatocyte intracellular lipids by resistin treatment suggests a multifactorial hepatic pathophysiological role of human resistin and makes resistin an attractive therapeutic target to inhibit. Moreover, these potentially lipotoxic effects of resistin should be examined further to determine whether oxidative stress is induced via lipotoxicity in hepatocytes. Resistin-mediated oxidative stress and enhanced inflammation has previously been reported in human endothelial cells. We did not find evidence of changes in AMP kinase expression or phosphorylation as a result of resistin treatment; therefore, AMP kinase-mediated alterations in hepatocyte oxidation levels and oxidative stress are not likely involved.

We wished to investigate potential mechanisms by which resistin mediates pathophysiological increases in expressed and secreted VLDL lipids and apoB by human hepatocytes. Our quantification of mRNA levels of the master genes initiating intracellular de novo lipogenesis (the SREBP1 and
SREBP2 transcription factors) showed significant increases in their expression and also in their target genes involved in intracellular FFA/triglyceride and cholesterol biosynthesis, respectively, after 24-hour resistin treatment.

Because cellular neutral lipids enhance intracellular apoB protein stability,8 the resistin-mediated increase in cellular neutral lipids, along with the reduction in intracellular insulin signaling, and stimulation of MTP activity can explain the enhanced apoB protein expression and secretion observed with hepatocyte human resistin treatment. All of these processes act to enhance apoB protein stability in hepatocytes, the main determinant of hepatocyte apoB protein levels.8

We wished to further examine the relevance of our hepatocyte studies with human resistin, delineated in the above discussion, in a more physiological human setting. We did this by using serum from obese and lean human subjects, with relatively higher and lower serum resistin concentrations, respectively, to stimulate hepatocyte apoB expression and secretion. We tested the serum from 36 human males of either lean (BMI, $\leq 25$ kg/m$^2$) or obese BMI (BMI, $> 30$ and $< 35$ kg/m$^2$) and WC. The individuals were otherwise healthy and were well characterized for lipid and lipoprotein variables. Our key finding here was that obese serum stimulated hepatocyte apoB cellular expression and secretion to a greater extent (by 5- and -fold, respectively) than lean serum. This is the first identification of a stimulatory effect of obese human serum on hepatocyte apoB. This indicated to us that factors in obese serum played a direct role in inducing greater hepatocyte apoB expression than lean serum.

Serum from obese individuals had 50% greater resistin levels than lean controls. We wished to test if the elevated serum resistin levels could count account for the greater hepatocyte apoB expression induced by obese human serum. When serum resistin was removed via immunoprecipitation using a polyclonal antibody against human resistin, the stimulatory effect of obese serum on hepatocyte apoB expression was reduced by 30%. This striking finding demonstrates that serum resistin has a quantitatively important role in mediating hepatocyte apoB production, and further demonstrates the potential of inhibitors of resistin as therapeutic targets to ameliorate dyslipidemia, particularly an elevation in hepatic apoB-containing lipoproteins, in humans.

Our control groups in the human serum hepatocyte apoB stimulation experiments included both lean serum-stimulated hepatocytes and hepatocytes incubated without serum (serum-free controls). Incubation of hepatocytes with lean serum, unexpectedly, had an inhibitory effect on apoB expression, compared to serum-free incubation of hepatocytes, and showed a further 50% decline in apoB expression on immunoprecipitation-antibody removal of serum resistin. Such an inhibitory effect of lean serum on apoB production should be investigated in future studies.

In conclusion, we have shown for the first time that human resistin induces hepatocyte oversecretion of VLDL apoB and lipids. At physiological concentrations, human resistin acts directly, in a dose-responsive manner, and much more potently than oleate, in stimulating hepatocyte secretion of increased numbers of smaller, more atherogenic VLDL particles, compared with control, untreated hepatocytes. Concomitantly, resistin elevates hepatocyte neutral lipid content, thereby explaining the positive association between plasma resistin levels and fatty liver and hepatic steatosis in humans. Associated resistin-mediated increases in de novo cellular cholesterol and triglyceride biosynthesis via the SREBP1 and SREBP2 pathways, respectively, can account for the increases in hepatic and secreted VLDL neutral lipids observed with human resistin treatment. Mechanisms by which human resistin induces hepatocyte apoB/VLDL oversecretion include stimulation of MTP activity and reduction in intracellular insulin signaling via both the PI3 kinase and MAP kinase pathways. A stimulatory effect on hepatic apoB secretion observed with obese human serum (with elevated resistin levels) incubation of hepatocytes was ameliorated by antibody removal of serum resistin. This indicates a quantitatively important role for serum resistin in mediating hepatic apoB production in humans.

Thus, in the present study, we have expanded our understanding of hepatic lipid and lipoprotein dysregulation in human obesity. In particular, we have further elucidated the mechanisms responsible for dyslipidemia development, particularly hepatic overproduction of VLDL, in obese humans, highlighting a key role for the adipose tissue-derived adipokine, resistin. Human resistin is, therefore, a potential therapeutic target for ameliorating the epidemic of dyslipidemia and ASCVD in human obesity, potentially reducing the high morbidity and mortality rates in these individuals.

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Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- Serum levels of the adipose tissue secreted signaling protein resistin are elevated in obese humans compared with lean healthy individuals.
- Elevated levels of resistin in humans are highly correlated with development of cardiovascular diseases (CVD), including coronary atherosclerosis.
- Although CVD risk factors, including insulin resistance and inflammation, develop in response to elevated resistin in multiple mouse models, translation of mouse data to humans has been difficult, possibly because of the low identity between the human and mouse forms of resistin. Hence, the link between resistin and CVD in humans has remained unclear.

**What New Information Does This Article Contribute?**

- We show for the first time a direct causal role of human resistin in elevating serum apolipoprotein (apo)B-containing lipoproteins (including very-low-density lipoproteins), a major CVD risk factor.
- We identify the cellular mechanisms by which human resistin stimulates hepatic overproduction of apoB-containing lipoproteins, which include enhancing hepatocyte apoB protein stability and reducing hepatocyte insulin signal transduction.

There is a strong positive association in humans between serum levels of the adipose tissue biomarker resistin and CVD. Although the causes of accelerated CVD in individuals with elevated resistin are not known, serum levels of resistin have been shown to be correlated with levels of atherogenic apoB-containing lipoproteins, a major CVD risk factor. This study was designed to determine whether human resistin directly influences the production of apoB-containing lipoproteins in human hepatocytes. Our results show that human resistin has a direct and potent stimulatory effect on human hepatocyte expression and secretion of apoB. The stimulatory effect of human resistin on apoB could be attributed to both enhanced cellular apoB stability, resulting from increased activity of microsomal triglyceride transfer protein (MTP), and to reduced expression of key proteins in the insulin signal transduction pathway (Akt, ERK). Although prior studies in rodent models have shown a direct causal role for resistin in elevated apoB levels, we show this effect for the first time with human resistin. We also elucidated the cellular mechanisms by which resistin stimulates hepatic apoB production. Thus, resistin is a novel and attractive therapeutic target for ameliorating dyslipidemia and CVD in humans.
Human Resistin Stimulates Hepatic Overproduction of Atherogenic ApoB-Containing Lipoprotein Particles by Enhancing ApoB Stability and Impairing Intracellular Insulin Signaling

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SUPPLEMENTAL MATERIAL

Methods:

Cell Culture. Cultured Hepatoma Cells: HepG2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). HepG2 cells were grown and maintained in 10% FBS-containing DMEM supplemented with 1% penicillin-streptomycin and 0.06% L-glutamine (584 mg/L) at 37°C, 5% CO2. During experiments in which HepG2 cells were treated with human recombinant resistin (Calbiochem, UK), the media was changed to 1% FBS-containing DMEM. Unless otherwise indicated, all experiments were performed in triplicate as three independent experiments. Cells were stimulated with human resistin at various doses (0, 5, 10, 25, 50 and 100 ng/mL) for 24 hours or with 50 ng/mL human resistin for various times (0, 2, 4, 8, 12, 24 and 48 hours). In separate experiments, cells were treated with the fatty acid, oleic acid (oleate) (100 μM) (Sigma, ON), with or without human resistin (50 ng/mL), for 24 hours. To mimic the effects of fatty-laden hepatocytes, as observed in individuals with fatty liver, in another set of experiments, HepG2 cells were pre-incubated with 500 µM of oleate for 24 hours before being stimulated with human resistin (50 ng/mL). In other experiments, cells were treated with 10 μM lactacystin (Cayman Chemical, Ann Arbor, MI) for 24 hours to assess intracellular proteosome-dependent apoB protein degradation. During HepG2 incubations with human serum, the media was replaced with DMEM plus 10% human serum from either lean (body mass index (BMI) ≤ 25 kg/m²) or obese (BMI > 30 kg/m² and < 35 kg/m²) male Multicultural Community Health Assessment Trial (M-CHAT) study participants2,3 (described below) for 24 hours.

Cell Culture. Primary Cells: Fresh wild-type rat and mouse primary hepatocytes were supplied by CellzDirect (Invitrogen, NC) in 6-well collagen coated plates. Upon arrival, the proprietary storage medium was removed and incubation medium (Williams E Medium, phenol red free, with incubation supplement pack, Gibco, NC) was added according to the manufacturers’ instructions. The cells were exposed to the incubation medium for 16 hours at 37°C with 5 % CO2 prior to human resistin (50 ng/mL) treatment for 24 hours. Cryopreserved plateable human hepatocytes, metabolism qualified, from multiple normal human donors, were supplied by CellzDirect (Invitrogen, NC). Upon arrival, the cells were plated according to the manufacturer’s instructions. That is, 4-8 million cells in 1 mL storage medium were added to 48 mL of warmed thawing medium (CHRM® Medium, Invitrogen, NC) and centrifuged at 100 x g for 10 minutes at room temperature. The resulting pellet was re-suspended in 4 mL of plating medium (Williams E Medium, phenol red free, with maintenance supplement pack and 10% FBS, Gibco, NC). Cells were stained with trypan blue (Sigma, Canada), counted with a haemocytometer and then seeded at 1 x 10^6 cells/well in a 6-well collagen coated plate (CellzDirect, Invitrogen, NC). The cells were incubated with the plating medium at 37°C with 5 % CO2 for 4 hours to allow the cells to adhere. The plating medium was then replaced with incubation medium and further incubated for 16 hours prior to human resistin treatment (50 ng/mL) for 24 hours.

Immunoprecipitation and Western Blots. Cell lysates, collected with RIPA buffer (50 mM Tris, 150 mM sodium chloride, 1% NP-40, 12 mM sodium deoxycholate, 3.5 mM SDS, pH 7.4 ) and protease inhibitor cocktail (Roche Diagnostics, QC), and media were immunoprecipitated for apoB100, apoCI, apoCIII, apoE, beta-actin, albumin, AMP-activated protein kinase (AMPK), phosphorylated AMP kinase (pAMPK(Thr172)), acetyl-CoA carboxylase (ACC), insulin receptor substrate (IRS)-2, extracellular signal-related kinase (ERK), phosphorylated ERK (pERK or p44/42 MAPK), phosphorylated on p44 residues Thr202/Tyr204 and p42 residues Thr185/Tyr187), Akt and serine and threonine phosphorylated Akt ((pAkt(Ser473)) and pAkt(Thr(308))) using Catch-and-Release immunoprecipitation columns and kits (Millipore, Billerica, MA) for immunocomplex pull-down. Immunoprecipitates containing equivalent amounts
of total protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes (BioRad, Hercules, CA) and immunoblotted using antibodies against the following proteins: apoB100 (human and rodent) (Santa Cruz, CA), apoC-I (Santa Cruz, CA), apoC-III (Santa Cruz, CA), apoE (Santa Cruz, CA), beta-actin (Sigma-Aldrich, St. Louis, MO), albumin (Santa Cruz, CA), IRS-2 (Millipore, CA), ERK and pERK (Cell Signaling Technology, MA), Akt, pAkt(Ser473) and pAkt(Thr308) (Cell Signaling Technology, MA) and AMPK and pAMPK(Thr172) (both antibodies were generous gifts generated in-house from Dr. Gregory Steinberg, McMaster University). Horseradish peroxidase–conjugated antibodies (BioRad, Hercules, CA) were used as secondary antibodies.\(^5\)\(^6\) Immunoreactive bands were visualized with a chemiluminescence kit (PerkinElmer Life Sciences, Waltham, MA). The blots were exposed to KODAK Biomax films, and the signal was quantified by densitometry using Quantity One version 4.6.7. software (Bio Rad, Hercules, CA).\(^5\)

**Real-Time Quantitative Reverse-Transcriptase (RT)-PCR Analysis.** Total RNA was isolated from cell lysates (RNeasy Mini Kit, Qiagen, Germantown, MD) and used as templates for cDNA synthesis (QuantiTech Reverse Transcription Kit, Qiagen, Germantown, MD). Quantitative real-time RT-PCR was performed using an Applied Biosystems 7300 Real Time PCR system (Carlsbad, CA), according to the manufacturer's instructions, and with the SYBR green master kit (Qiagen, Germantown, MD).\(^5\)\(^6\) Primers for the real-time RT-PCR internal control gene, cyclophilin A (sense, 5'-GTCAACCCCAACGTCTTCTC - 3'; antisense, 5'-TTTCTGTGTCTTGGGACCTTG -3'), were synthesized (IDT, Coralville, IA). Primers for succinate dehydrogenase (SDH) (another real-time RT-PCR internal control gene), apoB, microsomal triglyceride transfer protein (MTP), SREBP1, SREBP2, acetyl-coA carboxylase (ACC), HMG-CoA reductase, HMG-CoA synthase, squalene synthase (SS), the LDL receptor, PCSK9, fatty acid synthase (FAS), steroyl-CoA desaturase (SCD), diglyceride acyltransferase (DGAT)1 and DGAT2 were purchased (these proprietary sequences are not available) (Qiagen, Germantown, MD). The values reported for each mRNA were corrected to the cyclophilin A and SDH mRNA values.

**Oil-Red-O Staining.** Cells were stained with Oil-Red-O to examine the total amount of neutral lipid accumulation in the cells.\(^7\) Briefly, dishes were washed with cold phosphate-buffered saline and fixed in 10% neutral formalin. After 2 changes of propylene glycol, Oil-Red-O was added with agitation for 7 minutes, followed by washing in 85% propylene glycol. The dishes were then rinsed in distilled water and counterstained with hematoxylin. For each dish, 3 images were photographed, and a representative image is shown.

**Electron Microscopy.** The VLDL lipoprotein fraction was isolated from cell media via ultracentrifugation at a density of 1.006 g/mL using a Beckman Optima TL ultracentrifuge and 100,4 TLA rotor (Beckman Coulter, Brea, CA).\(^8\) The mean particle size for the VLDL fraction was then determined using the Hitachi 7000 electron microscope equipped with an AMT XR-60 digital camera. The fixation process for electron microscopy utilized 1% OsO4 in a phosphate buffer at pH 7.4 applied to the VLDL fraction for a 30 minute exposure.\(^9\) A small drop of this solution was placed on a 400 mesh copper grid coated with carbon film and allowed to stand for 3 minutes, or until the sample had dried. The fixed VLDL samples were placed on the electron microscope grid for viewing and digitizing. The captured images were taken at 75 KV using a beam current of 25 µA. Digital Images of the VDL were taken at 50,000X magnification. The mean particle size was then determined by importing the digital images into the NIH image J software program.
Lipid Measurements. Lipids from cell extracts obtained from 3-10 cm plates were pooled and quantified three times by gas chromatography (GC), as previously described. Briefly, cell extracts were incubated with phospholipase C (Sigma, ON) to remove polar head groups, then extracted in the presence of internal standard by the method of Folch. Extracted lipids were passed through a sodium sulfate column to remove aqueous contaminants and derivatized with Sylon BFT (Supelco, ON) to cap reactive hydroxyl and carboxyl groups. Derivatized lipids were dissolved in hexane and injected onto a Zebron ZB-5 column (Phenomenex, Torrance, CA) in an Agilent 6890 GC instrument.

Lipoprotein Profiles. Cell media from 6-10 cm plates was collected and concentrated using an Amicon Centriprep concentrator (50 K). Lipoprotein classes were separated from the concentrated media samples by fast protein liquid chromatography (FPLC) on a Superose 6 10/30 gel filtration column (Amersham, UK), followed by inline post-column reaction with either Infinity Triglyceride or Infinity Cholesterol reagent (Thermo Scientific, West Palm Beach, FL) and measurement of absorbance @ 500 nm. These methods have been adjusted from prior publications.

Cell Viability. Cell viability was determined using 0.4% trypan blue (Sigma-Aldrich, ON) staining and calculated using the following formula: % Cell Viability = Number of Unstained (Living) Cells / Total Number of Cells) x 100.

Microsomal Triglyceride Transfer Protein (MTP) Activity Assay. Cell monolayers were washed twice with ice-cold PBS and once with 5 mL of 1 mM Tris-HCl, pH 7.6, 1 mM EGTA and 1 mM MgCl₂ at 4 °C. Cells were then incubated for 2 minutes at room temperature in 5 mL of ice-cold 1 mM Tris-HCl, pH 7.6, 1 mM EGTA and 1 mM MgCl₂. The buffer was aspirated and 0.5 mL of the same buffer was added to cells. Cells were scraped and collected in ice-cold tubes, vortexed and centrifuged (SW55 Ti rotor, 50,000 rpm, 10 °C, 1 hour) and supernatants were used in the MTP assay using the MTP fluorometric activity assay kit (Chylos Inc., NY) as previously described. The triglyceride transfer activity of MTP is presented as %transfer/h/mg protein.

Human Subjects. Serum from participants recruited for the Multicultural Community Health Assessment Trial (M-CHAT) was used in the present investigation for stimulation of cellular and secreted apoB protein in human hepatocytes. The M-CHAT study consisted of a multiethnic cohort of healthy men and women, matched for ethnicity and BMI, between 30 and 65 years of age. Those who had a recent weight change (±2.2 kg in 3 months), had a previous diagnosis of CVD or significant comorbidity (such as HIV, an immunocompromised condition, type 1 diabetes mellitus), or had significant prosthetics or amputations were excluded. Those who were currently taking medications for CVD risk factors (i.e. lipid lowering, antihypertensive, or hypoglycemic medications) were also excluded. All participants provided informed consent. This study was approved by the Simon Fraser University Research Ethics Board. BMI was calculated as weight in kilograms divided by height in meters squared. Waist circumference (WC) was the average of 2 measurements taken against the skin at the point of maximal narrowing of the waist. Fasting blood samples were collected and immediately processed for total cholesterol, HDL cholesterol (HDL-C), triglycerides and glucose. All measurements were carried out in the same clinical laboratory with standard enzymatic procedures. For the present study, human serum was obtained from a multiethnic cohort (European and South Asian descent) of 36 exclusively male M-CHAT study subjects.

Resistin ELISA. The Quantikine Human Resistin ELISA kit was purchased from R&D Systems (Minneapolis, MN) to measure serum resistin concentrations in M-CHAT study participants from...
whom serum was used in hepatocyte apoB stimulation experiments. Serum resistin measurements were performed according to manufacturer’s instructions. In brief, serum was diluted 5-fold in the diluent supplied and incubated with the buffer supplied for 2 hours at room temperature in a 96 well plate. The plate was washed and resistin conjugate was added to each well for 2 hours. Following a second wash, substrate solution was added for 30 minutes and the reaction was completed by addition of a stop solution. The plate was read at 450 nm with a correction set at 570 nm. All samples were measured in duplicate.

**Resistin Immunoprecipitation.** To determine the effect of resistin antibody removal on human serum stimulation of cellular and secreted apoB in human hepatocytes, human resistin was immunoprecipitated from serum using Catch-and-Release immunoprecipitation columns and kits (Millipore, Billerica, MA). After equilibration of the columns with PBS, human serum was incubated with the beads in the column, along with resistin antibody (Santa Cruz, CA), PBS buffer and affinity ligand (supplied in the kits), with end over end rotation at 4 °C for 90 minutes, according to the manufacturer’s instructions. As a control, serum was also incubated with PBS buffer without resistin antibody or affinity ligand. The columns were centrifugated at 2000 g for 5 minutes and the flow-through was used for treatment of human hepatocytes for 24 hours. The columns were, thereafter, washed and eluted to confirm that resistin was captured when the resistin antibody was included in the immunoprecipitation incubations. After 24 hours of hepatocyte treatment with the resistin-immunoprecipitated serum, apoB protein in cell and media were measured via immunoprecipitation and Western blotting, as described above.

**Statistical Analysis.** Data were statistically analyzed using t-tests or one-way ANOVA, depending on the experimental conditions. All results are presented as mean ± SEM. Unless otherwise indicated, asterisks ((*) and (**)) indicate statistically significant differences (P < 0.05 and P < 0.01, respectively) compared with respective controls.
References:


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<td>23.33 ± 0.29</td>
<td>84.72 ± 2.14</td>
<td>1.18 ± 0.18</td>
<td>5.26 ± 0.21</td>
<td>3.21 ± 0.23</td>
<td>1.51 ± 0.09</td>
<td>5.26 ± 0.17</td>
</tr>
<tr>
<td>Obese</td>
<td>19</td>
<td>48.30 ± 2.28</td>
<td>31.84 ± 0.56*</td>
<td>105.79 ± 1.70*</td>
<td>1.90 ± 0.14*</td>
<td>5.15 ± 0.16</td>
<td>3.22 ± 0.13</td>
<td>1.06 ± 0.04*</td>
<td>5.49 ± 0.12</td>
</tr>
</tbody>
</table>

*P < 0.01 vs. Lean Subjects

**Online Table I.** Clinical and Biochemical Characteristics of Study Subjects. Metabolic characterization of individuals from whom serum was obtained for the present study. All individuals included in the present study are male. Human serum was added to DMEM (10% human serum in DMEM) for incubation with HepG2 cells to determine subsequent apoB100 protein expression (Figure 7.). Obese individuals in the current study had a BMI > 30 kg/m² and < 35 kg/m² and were of European or South Asian descent. Lean individuals had a BMI ≤ 25 kg/m² and were also of European or South Asian origin. Obese individuals had significantly greater serum triglyceride concentrations and significantly less serum HDL-C levels than lean controls.