Mechanisms mediating insulin resistance in transgenic mice overexpressing mouse apolipoprotein A-II.

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Abstract:

We previously demonstrated that transgenic mice overexpressing mouse apolipoprotein AII (apoAII) exhibit several traits associated with the insulin resistance (IR) syndrome, including increased atherosclerosis, hypertriglyceridemia, obesity, and IR. The skeletal muscle (SM) appeared to be the IR tissue in the apoAII transgenic (apoAIItg) mice. We now demonstrate a decrease in fatty acid (FA) oxidation in SM of apoAIItg mice, consistent with reports that decreased SM FA oxidation is associated with increased SM triglyceride accumulation, SM IR, and obesity. The decrease in FA oxidation is not due to decreased carnitine palmitoyltransferase-1 activity, since oxidation of palmitate and octanoate were similarly decreased. Quantitative RT-PCR analysis of gene expression demonstrated that the decrease in FA oxidation may be explained by a decrease in medium chain acyl-CoA dehydrogenase. We previously demonstrated that HDL from apoAIItg mice exhibit reduced binding to CD36, a scavenger receptor involved in FA metabolism. However, studies of combined apoAIItg and CD36 knockout mice suggest that the major effects of apoAII are independent of CD36. Rosiglitazone treatment significantly ameliorated IR in the apoAIItg mice, suggesting that the underlying mechanisms of IR in this animal model may share common features with certain types of human IR.
Introduction:

Alterations in both glucose and lipid metabolism are two consistent features of the insulin resistance syndrome (IRS) (1,2). Several studies have demonstrated that primary changes in fatty acid metabolism can lead to insulin resistance (3-8). While much has been learned about the insulin signaling pathways and the metabolism of both glucose and fatty acids, the underlying cause of most cases of IRS and type 2 diabetes is unknown (9,10). Whether primary defects in fatty acid metabolism or glucose metabolism underlie the majority of human cases of IRS and type 2 diabetes remains to be determined.

Studies using genetically altered mice have demonstrated that both the scavenger receptor, CD36, and the HDL associated protein, apolipoprotein A-II, alter fatty acid metabolism and insulin resistance (11-16). Furthermore, HDL is a ligand for the CD36 receptor, which raises the possibility of an HDL-CD36 interaction that affects fatty acid metabolism (15). Mice with a null mutation for apoAII exhibit increased sensitivity to insulin, with decreased plasma concentrations of triglycerides, FFA, and glucose (11). We previously demonstrated that increasing plasma concentrations of mouse apoAII in transgenic mice produced several aspects of the insulin resistance syndrome including, hypertriglyceridemia, obesity, and insulin resistance, as well as increased atherosclerotic lesion development (12,17-19). The skeletal muscle appeared to be the insulin resistant tissue in the apoAII transgenic mice, whereas the liver and adipose tissue in the apoAII transgenic mice appeared to respond normally (12). Compared to control mice, isolated soleus muscles from the apoAII transgenic mice exhibited decreased glucose uptake and increased triglyceride accumulation (12). We hypothesize that primary alterations in fatty acid metabolism in skeletal muscle initiate the insulin resistance and promote the development of obesity. In the present study we demonstrate that fatty acid oxidation is reduced in skeletal muscle of apoAII
transgenic mice, which could initiate the metabolic events leading to the skeletal muscle insulin resistance. We have also placed the apoAII transgene onto a CD36 knockout background and have demonstrated that plasma triglyceride and insulin concentrations are not markedly different in the apoAII transgenic mice whether or not functional CD36 receptors are present. In order to determine whether or not the mechanism underlying the insulin resistance in the apoAII transgenic mice resembles insulin resistance in humans, we were interested in determining if it would respond to thiazolinediones, a class of drugs used in treating insulin resistance in humans. We demonstrate that the insulin resistance in the apoAII transgenic mice quickly responds to treatment with rosiglitazone.

Research Design and Methods:

Animals. Transgenic mice containing multiple copies of the mouse apoAII gene, and CD36 null mice were derived as described previously (13,17). Homozygous apoAII transgenic and CD36 null mice were bred to produce 83 combined apoAII transgenic/CD36 null F2 progeny. All animals were housed 3-4 to a cage, maintained at 24 °C on a 12 hour light-dark cycle, and provided Harlan-Teklad rodent chow (6% fat) and water ad libitum. The care of the mice, as well as all procedures used in this study were done in accordance with NIH animal care guidelines. The animals were housed at the upper approved temperature limit (24 °C), which appears to be more appropriate for mice (20,21). With the exception of apoAII transgenic mice generated in the cross to the CD36 null mice described above, all apoAII transgenic animals used in the present study were males, homozygous for the apoAII transgene. Age matched male C57BL/6 mice were used as the controls.
Lipid analyses. Plasma was collected from mice that were fasted overnight and bled 2-3 hours after the beginning of the light cycle from the retro-orbital plexus under isoflorane anesthesia. Total cholesterol, HDL cholesterol, triglycerides, and FFA concentrations were determined as described previously (17,18). HDL was isolated by precipitation of VLDL and LDL with heparin and manganese chloride (22). Each lipid determination was measured in triplicate. An external control sample with known analyte concentration was run in each plate to ensure accuracy.

Hepatic and skeletal muscle glycogen content. Tissue samples were isolated 2-3 hours after the beginning of the light cycle from animals that had been allowed ad libitum access to food and water. After homogenization in ddH2O, aliquots were transferred to an acetate buffer solution containing 5x10^{-5} g α-glucosidase (Sigma, cat #A7420) and 2.7 ul α-amylase (Sigma, cat #A4268) per ml of buffer solution. Aliquots of homogenates were also transferred to acetate buffer solution containing no enzymes, to serve as a sample blank. A standard curve was generated with a serial dilution of glucose. After addition to the buffer solution the tubes were incubated at 37° C for 10 minutes, centrifuged in a microcentrifuge for 5 min at 3000 rpm and 170 ul of the supernatant, in triplicate, was transferred to a 96 well plate and read at 490 nm is a Molecular Devices Spectramax plus microplate reader.

Skeletal muscle fatty acid oxidation. Following an overnight fast, mice were anesthetized with pentobarbital (50mg/kg body wt) and the soleus muscle was isolated under a dissecting microscope. The muscles were weighed and immediately incubated for 40 min in Krebs-Henseleit buffer under 95% O2:5% CO2, that contained 5.5 mM glucose, 3% BSA, 1 mM palmitate and either [14C-U]-palmitic acid (2.5 uCi/ml) or [14C-U]-octanoic acid (2.5 uCi/ml). Rates of FA oxidation were assessed by determining radioactivity in 14CO2 trapped in hyamine hydroxide as described previously (23). Palmitate (1mM) was used as the unlabeled exogenous fatty acid in experiments.
with both $[^{14}\text{C}}\text{-U}]$-palmitic acid and $[^{14}\text{C}}\text{-U}]$-octanoic acid tracers. Octanoate, which is not normally present in high concentrations has markedly different effects on metabolism compared to long chain fatty acids (24-26). By using palmitic acid as the unlabeled fatty acid substrate in both sets of experiments the physiologic effects of the exogenous fatty acid substrate was the same in both groups allowing us to observe differences in metabolism of the tracer quantities of radiolabeled palmitate and octanoate under the same conditions.

**Rosiglitazone treatment.** One group of 14 apoAII transgenic mice were fasted overnight and bled 2-3 hours after the beginning of the light cycle from the retro-orbital plexus under isoflorane anesthesia. The mice were allowed to recover for two weeks and were then placed on a diet that contained rosiglitazone (4 g/kg diet). After one week of ad libitum access to the rosiglitazone diet the mice were again fasted and bled. Plasma lipids, insulin, and glucose concentrations were determined before and after rosiglitazone treatment. In order to determine the effect of rosiglitazone treatment on skeletal muscle fatty acid oxidation, another group of 9 apoAII transgenic mice were placed on the rosiglitazone diet for 1 week. The animals were fasted overnight, and rates of fatty acid oxidation ($^{14}\text{C}$-palmitate) were determined in isolated soleus muscles as described above, and were compared to rates of oxidation in another group of age matched apoAII transgenic male mice that received the identical diet without rosiglitazone.

**Insulin and glucose assays.** Plasma insulin concentrations were determined in duplicate by ELISA using kits from Crystal Chem. Inc. (cat. #IVSKR020). The intra- and inter-assay precision for the insulin assays are 3.5% and 6.3%, respectively. The minimum detectable level of mouse insulin is 156 pg/ml. Plasma glucose concentrations were determined in triplicate using a commercially available kit (Sigma, #315-100). In experiments where glucose concentrations were
determined, plasma was isolated within 15 minutes after bleeding in order to minimize glucose metabolism by erythrocytes.

**Western blot analysis of CD36, apoAII, and apoAI.** CD36 was extracted from skeletal muscles of control and apoAII transgenic mice following an overnight fast. Briefly, aliquots of skeletal muscle were homogenized in ice cold phosphate buffered saline containing 1% Igepal CA-630 (Sigma cat # I3021), 0.5% sodium deoxycholate (Sigma cat # D6750), 0.1% SDS(Sigma cat # L4509), 1x10^{-4} g/ml PMSF (Sigma cat # P7626), 30ul/ml aprotinin (Sigma cat # A6279), and 1x10^{-4} M sodium orthovanadate (Sigma cat # S6508). Extracted tissue proteins were separated on 4-20%, 1mm thick, tris-glycine gels (Novex). CD36 was identified by western blotting using the Cascade Bioscience Primary CD36 antibody (cat # ABM5525) diluted 1:1000 in 5% milk-PBST (phosphate buffered saline with Tween) and quantitated by chemiluminescent detection (Amersham) and densitometry. Plasma apoAII and apoAI concentrations were determined using the same type of gels and chemiluminescent detection followed by densitometry. Aliquots of a pooled mouse plasma sample were included on each gel in order to allow normalization of the results among samples from different gels. The anti mouse apoAII and apoAI antibodies were from Biodesign International (Camarillo, CA). Previously determined average apoAI (119 mg/dl) and apoAII (15 mg/dl) concentrations in plasma from C57BL/6 mice were used as the reference value to convert densitometric units to mg/dl (27).

**Expression of heart type fatty acid binding protein (H-FABP), fatty acid transport protein-1 (FATP-1), and medium chain acyl CoA dehydrogenase (MCAD) in skeletal muscle of apoAII transgenic and control mice.** Total mouse muscle RNA was isolated from four individual control and four apoA-II transgenic male mice using Trizol reagent (Invitrogen) according to the
manufacturers protocol. One µg of total RNA was reverse transcribed using Oligo dT and Superscript-III reverse transcriptase (Invitrogen).

Quantitative RT-PCR was performed using an ABI Prizm 7700 Sequence Detection System (Applied Biosystems) and SYBR Green detection (SYBR Green Taq ready mix, Sigma). cDNA sequences for the analyzed genes were obtained from the gene bank and primers were designed using the PrimerQuest software (IDT Technologies). The primer sequences were as follows: mFABP forward 5’-AACGGGCAGGAGAC AACACTAACT-3’, reverse 5’-TCATAAGTCCGAGTGCTCA CCACA-3; mMDC forward 5’-TCGGTGAAGGAGCGGTTTCAAGA-3’, reverse 5’-AAACTCCTTGGTGTCCACTAGCA-3’; mFATP1 forward 5’-ACAGCCAGTTGG ACCCTAACTCAA-3’, reverse 5’-TGGATCTTGAAGGTGCTGTGTA-3’. Primer sequences for the housekeeping gene, β2 microglobulin (β2M) were described previously (28). Primers were designed for their product to span at least 2 exons, have a melting temperature of 60°C and verified using BLAST search against the mouse genome database. Correct sizes of the PCR products (amplicons) were verified by agarose gel electrophoresis. Additional melting curve analysis of each PCR product was performed to confirm the presence of a single amplicon in the PCR reaction. Serial dilutions of the pooled samples were used to construct the standard curve and determine the real-time PCR efficiency for each primer pair using the ABI Prizm 7700 software. Each individual mouse cDNA sample was analyzed separately by obtaining the relative expression values from the constructed standard curve and correcting for the β2M expression. The final data are expressed as relative expression of a respective gene in the apoA-II group versus the control group (set as 100%).
Results:

We previously observed that mice overexpressing mouse apoAII exhibited not only altered lipoprotein profiles but also insulin resistance (12). ApoAII plasma concentrations differed between males and females and between mice heterozygous and homozygous for the transgene (table 1). Clearly, there is a dose dependent effect of apoAII expression on plasma concentrations of cholesterol, triglycerides, free fatty acids, glucose and insulin (table 1). Our present studies have been conducted using male mice homozygous for the apoAII transgene because of the higher levels of apoAII, making it easier to detect apoAII effects on traits relevant to glucose and fatty acid metabolism.

We previously reported that skeletal muscle appears to be the insulin resistant tissue in the apoAII transgenic mice (12). Those aspects of the phenotype that we had examined with respect to hepatic metabolism, suggested that the livers in the apoAII transgenic mice were responding normally to the increased plasma insulin concentrations. Since hepatic and skeletal muscle glycogen content is an indicator of insulin responsiveness (29), we determined the glycogen content of these tissues in apoAII transgenic and control mice. Hepatic glycogen mass was significantly increased in the apoAII transgenic mice, while skeletal muscle glycogen was significantly reduced (Figs. 1A and 1B). These results are consistent with a normal response of the liver to the elevated insulin levels in the apoAII transgenic mice, and with skeletal muscle insulin resistance.

Since altered fatty acid oxidation in skeletal muscle is believed to result in skeletal muscle insulin resistance, we determined rates of oxidation of U-14C-palmitic acid in soleus muscles isolated from animals that had been fasted overnight. Oxidation of palmitic acid to CO₂ was reduced approximately 30% in the skeletal muscle from the apoAII transgenic mice compared to controls (Fig. 2A). Carnitine palmitoyltransferase-1 (CPT-1) is necessary for the transport of long
chain fatty acids into the mitochondria and is generally regarded as the rate-limiting step in fatty acid oxidation (30). Decreased CPT-1 activity has been linked to skeletal muscle insulin resistance, increased skeletal muscle TG content and the development of obesity, all phenotypes of the apoAII transgenic mouse (31-33). In order to determine if changes in CPT-1 activity could account for the lower rates of fatty acid oxidation in skeletal muscle from the apoAII transgenic mice, the experiments were repeated using the short chain fatty acid, octanoate. Octanoate does not require CPT-1 for transport into the mitochondria. The rates of oxidation of octanoate were also decreased approximately 25% in the skeletal muscle of the apoAII transgenic mice, suggesting that the lower rate of fatty acid oxidation in skeletal muscle is not due to decreased activity of CPT-1 (Fig. 2B).

While CPT-1 is generally regarded as the rate limiting step in the β-oxidation of long chain fatty acids, β-fatty acid oxidation is a complex process involving many different steps. We examined the expression of three genes whose products are known to affect the β-oxidation of fatty acids by acting at diverse points in the fatty acid metabolic pathway. Medium chain acyl CoA dehydrogenase (MCAD) is a mitochondrial enzyme that catalyzes the initial reaction in the fatty acid β-oxidation cycle (34,35). Heart type fatty acid binding protein (H-FABP) is a cytosolic protein important in the intracellular transport of fatty acids with effects on triglyceride accumulation and oxidation (36-38). Fatty acid transport protein-1 (FATP-1) is an integral plasma membrane protein involved with fatty acid uptake into the cell, and which also has been reported to have acyl CoA synthase activity (39-41). Consistent with the decreased fatty acid oxidation observed in skeletal muscle of the apoAII transgenic mice, expression of MCAD was significantly decreased, and there was a trend towards decreased expression of both H-FABP and FATP-1 (Fig 3).
As described above, CD36 appears to be a good candidate for mediating the effects of apoAII on skeletal muscle fatty acid metabolism. In order to determine if the content of CD36 in skeletal muscle was altered in the apoAII transgenic mice, total protein was extracted from aliquots of skeletal muscle from fasted animals and separated by polyacrylamide gel electrophoresis. Western blot analysis demonstrated that skeletal muscle CD36 content was not significantly different between control and apoAII transgenic mice (Fig. 4).

In order to determine if CD36 alters plasma concentrations of apoAII in mice, we determined plasma concentrations of apoAII in the CD36 knockout and control mice. ApoAII was increased in the plasma of CD36 null mice while apoAI concentrations were unchanged, providing support for an interaction between CD36 and apoAII (Fig. 5). In in vitro HDL binding studies using CD36 transfected COS cells, we previously demonstrated that HDL from the apoAII transgenic mice exhibit significantly reduced binding to the CD36 receptor, and that this decreased binding was associated with a decrease in cholesterol exchange (42).

In order to investigate the in vivo effects of CD36 on the apoAII transgenic phenotype, apoAII transgenic mice on the CD36 null background were derived as described in the methods. The effects of the apoAII transgene were not markedly different, whether or not the CD36 receptor was present. Plasma concentrations of apoAII correlated with total and HDL cholesterol (Fig. 6), free fatty acids and triglycerides (Fig. 7), and glucose and insulin (Fig. 8), in both CD36 wild type and CD36 null mice. We conclude that the major effects of apoAII on plasma lipids and insulin resistance in our mice are not mediated by CD36.

In order to determine if the insulin resistance in the apoAII transgenic mice would respond to thiazolidinediones, a class of drugs used in the treatment of human insulin resistance, we treated the mice with rosiglitazone. After only one week of treatment, rosiglitazone significantly reduced
plasma concentrations of triglycerides, FFA, glucose, and insulin (Table 2), effects similar to those observed in humans (43). There was also a decrease in HDL cholesterol after rosiglitazone treatment, however, no significant changes in plasma concentrations of apoAI or apoAII were observed (Table 2).

In order to determine if skeletal muscle fatty acid oxidation was altered by rosiglitazone treatment we placed another group of apoAII transgenic mice on rosiglitazone for one week and compared rates of palmitate oxidation in isolated soleus muscles from these animals to rates of oxidation in muscles from a separate group of age matched apoAII transgenic mice which did not receive rosiglitazone. Following rosiglitazone treatment there was a trend towards increased fatty acid oxidation (Fig 9).

Discussion:

We previously demonstrated that transgenic mice overexpressing the HDL apolipoprotein, apoAII, exhibit several traits associated with the insulin resistance (IR) syndrome (12). In examining the basis for the insulin resistance in the apoAII transgenic mice, we had determined that the skeletal muscle was the insulin resistant tissue (12). In the present study we demonstrate that fatty acid oxidation is decreased in the skeletal muscle of the apoAII transgenic mice (Fig. 2), and that this decrease in oxidation is unlikely due to changes in carnitine palmitoyltransferase-1, but rather to the step in oxidation catalyzed by MCAD (Fig. 3). We previously demonstrated that HDL enriched in apoAII binds less efficiently to CD36, a scavenger receptor with known effects on fatty acid and glucose metabolism (42). In the present study we demonstrate that overexpression of
apoAII does not alter CD36 protein concentrations in skeletal muscle (Fig. 4), however, knocking out CD36 does increase plasma concentrations of apoAII (Fig. 5). To further evaluate the role of CD36 in mediating the effects of apoAII overexpression, we generated 83 progeny from an intercross of CD36 null and apoAII transgenic mice. The apoAII transgenic phenotype was not markedly different whether or not CD36 was expressed (Figs. 6-8). We also demonstrate that the insulin resistance phenotype in the apoAII transgenic mice is markedly ameliorated by only one week of treatment with rosiglitazone (Table 2).

Plasma insulin concentrations are 2.5-3 times higher in the apoAII transgenic mice. Insulin is a regulator of the glycogen content in both liver and skeletal muscle (29). Insulin stimulates the conversion of glucose to glucose 6-phosphatase, which then undergoes isomerization to glucose 1-phosphate and is incorporated into glycogen by the enzyme glycogen synthase, the activity of which is also stimulated by insulin. Insulin also inhibits phosphorylase, which decreases glycogen breakdown. The increased concentration of glycogen in the livers of the apoAII transgenic mice is consistent with a normal response by the liver to the increased plasma insulin concentrations (Fig. 1). In contrast, the decreased skeletal muscle glycogen content in the apoAII transgenic mice (Fig. 1), even in the presence of the increased plasma insulin concentrations in these animals, is consistent with skeletal muscle insulin resistance.

Increased plasma FFA concentrations have been hypothesized to contribute to insulin resistance by increasing utilization of fatty acid by muscle, as a consequence of the concentration dependent uptake of FFA (4, 44). Increased fatty acid oxidation is then believed to inhibit glucose oxidation and glycolysis, with subsequent inhibition of glucose uptake. On the other hand, some evidence suggests that the basis of skeletal muscle insulin resistance in obesity may actually be an impaired capacity for utilization of plasma FFA (45-48). Utilization of FFA for energy by skeletal
muscle has been reported to be reduced in obesity. There is evidence that impaired oxidation of fat may be a contributing factor to weight gain (32,33). The activity of carnitine palmitoyltransferase-1 (CPT-1) in muscle has been negatively correlated with visceral fat mass, and rates of FFA uptake correlated with CPT-1 activity (31). These studies suggest that decreased oxidative capacity and utilization of FFA by skeletal muscle can result in skeletal muscle insulin resistance and increased adiposity. Skeletal muscle fatty acid oxidation is reduced in the apoAII transgenic mice (Fig. 2), consistent with studies that have linked decreased fatty acid oxidation to the development of skeletal muscle insulin resistance and obesity, the phenotype we observe in the apoAII transgenic mice (12). However, the reduced rates of oxidation in the apoAII transgenic mice do not appear to be due to lower CPT-1 activity, since the oxidation of octanoate, which does not require CPT-1 for transport into mitochondria, was also reduced (Fig. 2). We used quantitative RT-PCR to examine the expression of three genes important in fatty acid metabolism and which act at diverse points in the fatty acid metabolic pathway; MCAD, H-FABP, and FATP-1. The expression of MCAD was significantly decreased in skeletal muscle of apoAII transgenic mice compared to controls (Fig. 3). MCAD is a member of a family of mitochondrial dehydrogenases that catalyze the initial reaction in the fatty acid β-oxidation cycle (34,35). MCAD shows greatest activity for fatty acid substrates that are from 8-12 carbons in chain length. Decreased activity of MCAD would affect the oxidation of the 8-carbon fatty acid octanoate, as well as the oxidation of the 16-carbon palmitate, after the length of the original palmitate was shortened through successive cycles of β-oxidation. We also observed a trend towards decreased expression of both H-FABP and FATP-1 (Fig. 3). H-FABP is a cytosolic protein important in the intracellular transport of fatty acids, with effects on triglyceride accumulation and oxidation (36-38). Fatty acid transport protein-1 (FATP-1) is an integral plasma membrane protein involved with fatty acid uptake into the cell, and which also has been reported to
have acyl CoA synthase activity (39-41). Decreased expression of MCAD is sufficient to explain the results of our fatty acid oxidation experiments. Whether decreased expression of H-FABP and FATP-1 contribute to the reduced fatty acid oxidation, or merely represent a response to the reduced rates of oxidation as fatty acids and their metabolites accumulate in the cell, remains to be determined.

HDL is a ligand for CD36, a scavenger receptor that has been demonstrated to be involved in fatty acid and glucose metabolism (13,14). We had previously demonstrated that HDL from the apoAII transgenic mice exhibited reduced binding to the CD36 receptor, and decreased cholesteryl ester exchange as a result (42). Also, genetic analysis of a Mexican-American study population indicated a locus on chromosome 7 (lod score 3.0) over the CD36 gene that was linked to plasma concentrations of apoAII (data not shown). Therefore, CD36 appeared to be a reasonable candidate for mediating the effects of altered HDL upon skeletal muscle fatty acid metabolism. There were no significant differences in the CD36 content of skeletal muscle from the apoAII transgenic mice compared to controls (Fig. 4), however plasma concentrations of apoAII were significantly increased in the plasma of the CD36 knockout mice (Fig. 5). This effect of CD36 on apoAII concentrations is supported by the association of the CD36 gene locus with plasma apoAII concentrations in the Mexican-American study population described above. An earlier study also demonstrated that CD36 knockout mice had a significant increase in plasma HDL cholesterol, as well as a shift to larger HDL particles, consistent with the changes in HDL observed with increasing apoAII concentrations in our transgenic mice. (13). The mechanism through which CD36 increases plasma HDL cholesterol and apoAII concentrations in these mice is not known, but may involve changes in normal HDL metabolism related to cholesteryl ester exchange mediated via the CD36 receptor, which ultimately affects clearance/metabolism of apoAII enriched HDL. In order to
examine the role of CD36 in mediating the apoAII transgenic phenotype *in vivo*, we derived combined apoAII transgenic/CD36 null mice. The effects of apoAII on plasma lipids, cholesterol, insulin and glucose, were similar whether or not CD36 was present (Figs. 6-8). Overall these results suggest that there is an HDL-CD36 interaction that has effects on HDL metabolism, however, the primary effects of apoAII in the transgenic mice do not appear to be mediated by CD36.

In order to further assess the similarity between the insulin resistance phenotype in the apoAII transgenic mice and certain types of human insulin resistance, we treated the mice with the insulin sensitizing thiazolidinedione, rosiglitazone. After only one week of treatment, rosiglitazone significantly reduced plasma concentrations of triglycerides, FFA, glucose, and insulin (Table 1), consistent with effects observed in humans (43). We also observed a significant reduction in HDL cholesterol, however, plasma concentrations of apoAI and apoAII were not significantly changed (Table 1). In humans, by contrast, most studies report an increase in HDL cholesterol in response to thiazolidinedione treatment (49,50). The effect we observed with rosiglitazone on HDL cholesterol levels in the apoAII transgenic mice is consistent with a previous study that demonstrated a decrease in HDL cholesterol when diabetic LDL receptor deficient mice were treated with troglitazone (51). Although the mechanism responsible for the different effects of thiazolidinediones on plasma HDL concentrations between mice and humans is not known, it most likely reflects differences in HDL metabolism between the two species (as discussed below). In a separate series of experiments rates of fatty acid oxidation in soleus muscles isolated from mice that were treated with rosiglitazone were compared to rates of oxidation in another group of apoAII transgenic mice that did not receive the drug. There was a trend towards increased fatty acid oxidation after rosiglitazone treatment (Fig. 9). The failure to reach statistical significance could reflect differences in basal rates of oxidation between the two groups of mice used in the study. In
these experiments we could not use the same group of mice to compare pre-and post drug effects, as we were able to do in examining the changes in plasma lipids, glucose, and insulin in the initial experiment.

The effects of overexpressing mouse apoAII on fatty acid and glucose metabolism does not appear to be a non-physiologic response that is observed only with markedly elevated plasma apoAII concentrations. Earlier genetic studies from our laboratory demonstrated a link between the apoAII gene locus and plasma concentrations of apoAII and free fatty acids in common inbred strains of mice (52). In our apoAII transgenic mice, the effects of apoAII in both sexes, and between mice heterozygous and homozygous for the apoAII transgene, demonstrate a dose response relationship with plasma apoAII concentrations (table 1). This dose response effect of apoAII was also observed in the progeny from the apoAII transgenic/CD36 null cross (Figs. 6-8) Furthermore, mice which completely lack apoAII exhibit increased insulin sensitivity, with decreased plasma concentrations of triglycerides, free fatty acids, and glucose (11). Thus, the effects of mouse apoAII on plasma lipids and insulin resistance have been demonstrated over the entire range of plasma apoAII concentrations.

The present study was performed using mice that express a transgene for mouse apoAII. Studies in mice that express a transgene for human apoAII have found similarities as well as differences with respect to the effects of apoAII in the mouse, as described in a recent comprehensive review (53). Most (54-61), but not all (62-66) of these studies have demonstrated that increasing human apoAII in the mouse resulted in an increase in plasma triglycerides and non-HDL cholesterol, consistent with the effects observed in the present study. However, unlike the transgene for mouse apoAII, human apoAII does not increase HDL cholesterol or appear to affect insulin resistance or obesity in these mouse models (67). Sequence differences between mouse and
human apoAII are likely to account for some of these differences in HDL metabolism (53). Although human apoAII does not affect insulin resistance and obesity in mice, several studies have demonstrated an effect of apoAII on insulin resistance and increased fat mass in humans (68-70,72). Therefore, failure to observe these effects with human apoAII in mice, may be due to species specific interactions with other apolipoproteins, receptors, enzymes, and lipid transfer proteins that are involved in HDL metabolism.

Many studies have demonstrated the usefulness of the mouse as an experimental model to investigate aspects of lipoprotein metabolism relevant to human physiology. Still, care must be exercised when extrapolating the results of any animal study to humans. The phenotype in the apoAII transgenic mice resembles many aspects of the insulin resistance syndrome (IRS) in humans, including increased adiposity, insulin resistance, and hypertriglyceridemia. However, IRS in humans is associated with decreased plasma HDL cholesterol, while the apoAII transgenic mice have increased HDL cholesterol. Part of this difference in HDL cholesterol probably relates to differences in human versus mouse apoAII, as discussed above. When a transgene for human apoAII was expressed in mice, plasma HDL cholesterol concentrations were reduced and the HDL became smaller in size (57,64). Mouse apoAII appears to have the opposite effect, since increasing mouse apoAII in our studies results in increased HDL cholesterol and larger particles, while apoAII null mice have HDL that are smaller in size. Although overexpression of mouse apoAII had the opposite effect on total HDL cholesterol compared to human apoAII, both increased triglycerides and non-HDL cholesterol. A similar situation apparently exists with thiazolidinedione treatment, which appears to ameliorate insulin resistance in both mice and humans with a decrease in plasma concentrations of triglycerides, FFA, glucose and insulin, while total HDL cholesterol is generally increased in humans and decreased in mice. Thus, because of differences in HDL metabolism
between mice and humans, effects on total HDL cholesterol can be different, while other effects on lipoprotein metabolism and insulin resistance may be similar. Another explanation for this apparent difference in effects on HDL cholesterol between human metabolic syndrome and our mouse model, relates to using total HDL cholesterol as an indication of metabolically relevant changes in HDL. In both mice and humans HDL are not a single homogeneous type of lipoprotein, but consist rather of several types of particles that differ in size and composition, and which have been demonstrated to have different metabolic effects. Changes in total HDL cholesterol may not reflect important changes in various sub-fractions of HDL, which may be the ones that are more relevant to the traits under investigation. This is supported by the observation that HDL from the apoAII transgenic mice are not as protective as HDL from control mice in preventing the oxidation of LDL in a cell culture model of the artery wall (19). Evidently the sub-fraction of HDL important for this protective effect is reduced in the apoAII transgenic mice even though total HDL cholesterol is increased.

Studies in humans also have demonstrated links between apoAII and increased atherosclerosis, type 2 diabetes, plasma concentrations of triglycerides and FFA, and visceral obesity (52,68-72). The similar effects of apoAII in our transgenic mice and in humans, and the observation that the insulin resistance in the apoAII transgenic mice responds to treatment with rosiglitazone, suggest similarities to some types of human insulin resistance and type 2 diabetes. Thus, the apoAII transgenic mouse appears to be an interesting animal model for further research.
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References:


Figure Legends:

Figure 1. Hepatic and skeletal muscle glycogen content. The concentration of glycogen in liver (Panel A) and skeletal muscle (Panel B) from apoAII transgenic and control mice were determined. Animals were sacrificed 3 hours after the beginning of the light cycle, having been allowed ad libitum access to food throughout the dark cycle. Data represent the mean ± the SEM for 4 animals in each group. * indicates values significantly different from control mice, p<0.05.

Figure 2. Skeletal muscle fatty acid oxidation. Oxidation of 14C-labeled palmitate (Panel A) and 14C-octanoate (Panel B) were determined in soleus muscles isolated from control and apoAII transgenic mice that had been fasted overnight. Data represent the mean ± the SEM for 5 animals in each group. * indicates values that are significantly different from control mice, p<0.05.

Figure 3. Expression of medium chain acyl CoA dehydrogenase (MCAD), heart type fatty acid binding protein (H-FABP) and fatty acid transport protein (FATP) in skeletal muscle. Expression of MCAD, H-FABP, and FATP was determined in skeletal muscle of control and apoAII transgenic mice by quantitative real time PCR using SYBR Green detection as described in the methods section. Data are the mean ± SEM for 4 animals in each group, presented as the percentage of expression observed in the control mice. * indicates values that are significantly different from control, p<0.05.

Figure 4. Skeletal muscle CD36 content. Following an overnight fast, skeletal muscle from control, apoAII transgenic (apoAII), and CD36 null (CD36ko) mice was dissected under a
microscope. Total protein was isolated and subjected to PAGE followed by western blot analysis and densitometric scanning. The relative concentrations of CD36 in each sample are expressed in relative densitometry units. The data represent the mean ± the SEM for 3 animals in each group. CD36 null mice were included as a negative control. As expected CD36 was not detected in this group.

Figure 5. Plasma apoAII and apoAI concentrations in CD36 null mice. Following an overnight fast, mice were anesthetized, and blood obtained from the retro-orbital plexus. Aliquots of plasma were subjected to PAGE followed by western blot analysis for apoAII and apoAI. The relative concentrations of apoAII (Panel A) and apoAI (Panel B) were then determined by densitometric scanning. The relative concentrations of the apolipoproteins in each sample are expressed in relative densitometry units. The data represent the mean ± the SEM for 5 animals in each group. *indicates values that are significantly different from control, p<0.05.

Figure 6. Effects of plasma apoAII concentrations on total and HDL cholesterol in control and CD36 null mice. The apoAII transgene was bred onto the CD36 null background, producing 83 F2 progeny. From the F2 progeny 21 mice (11 females, 10 males) were wild type for CD36 (CD36 wt) and 38 (23 females, 15 males) were homozygous for the CD36 null transgene (CD36ko). Following an overnight fast, mice were bled from the retro-orbital plexus under isoflorane anesthesia and plasma concentrations of total cholesterol, HDL cholesterol, and apoAII were determined as described in Methods. Plasma total cholesterol is plotted against plasma apoAII concentrations in the upper section of the figure for both CD36 wt (left side) and CD36 null (right side) mice. Plasma HDL is plotted against apoAII concentrations in the lower portion of the figure.
Cholesterol concentrations are expressed in mg/dl and apoAII concentrations are expressed in relative densitometry units. Linear regression analysis was performed using Statview statistical analysis software (Abacus Concepts).

Figure 7. Effects of plasma apoAII concentrations on triglycerides and free fatty acids in control and CD36 null mice. The apoAII transgene was bred onto the CD36 null background, producing 83 F2 progeny. From the F2 progeny 21 mice (11 females, 10 males) were wild type for CD36 (CD36 wt) and 38 (23 females, 15 males) were homozygous for the CD36 null transgene (CD36 null). Following an overnight fast, mice were bled from the retro-orbital plexus under isoflorane anesthesia and plasma concentrations of triglycerides, free fatty acids, and apoAII were determined as described in Methods. Plasma triglyceride concentrations are plotted against plasma apoAII concentrations in the upper section of the figure for both CD36 wt (left side) and CD36 null (right side) mice. Plasma free fatty acids are plotted against apoAII concentrations in the lower portion of the figure. Triglyceride and free fatty acid concentrations are expressed in mg/dl and apoAII concentrations are expressed in relative densitometry units. Linear regression analysis was performed using Statview statistical analysis software (Abacus Concepts).

Figure 8. Effects of plasma apoAII concentrations on glucose and insulin in control and CD36 null mice. The apoAII transgene was bred onto the CD36 null background, producing 83 F2 progeny. From the F2 progeny 21 mice (11 females, 10 males) were wild type for CD36 (CD36 wt) and 38 (23 females, 15 males) were homozygous for the CD36 null transgene (CD36ko). Following an overnight fast, mice were bled from the retro-orbital plexus under isoflorane anesthesia and
plasma concentrations of glucose, insulin, and apoAII were determined as described in Methods. Plasma glucose concentrations are plotted against plasma apoAII concentrations in the upper section of the figure for both CD36 wt (left side) and CD36 null (right side) mice. Plasma insulin concentrations are plotted against apoAII concentrations in the lower portion of the figure. Glucose concentrations are expressed in mg/dl, insulin in pg/ml, and apoAII concentrations are expressed in relative densitometry units. Linear regression analysis was performed using Statview statistical analysis software (Abacus Concepts).

Figure 9. Effects of rosiglitazone treatment on skeletal muscle fatty acid oxidation. Rosiglitazone was administered in the diet (4 mg/kg) to a group of apoAII transgenic male mice for one week (+rosi). A control group of age matched apoAII transgenic male mice received the identical diet without rosiglitazone (-rosi). Oxidation of $^{14}$C-labeled palmitate to CO$_2$ was determined in soleus muscles isolated from mice that had been fasted overnight. Data represent the mean ± the SEM for 9 animals in each group.
Table 1. Changes in plasma concentrations of total cholesterol, HDL cholesterol, triglycerides, free fatty acids (FFA), glucose and insulin correlate with plasma apolipoprotein AII (apoAII) concentrations in the transgenic mice. Five month old C57BL/6 (C57) controls, heterozygous apoAII transgenics (het) and homozygous apoAII transgenics (homo) were fasted overnight prior to bleeding. All values are expressed as the mean ± the SEM in mg/dl except for insulin concentrations that are expressed in pg/ml.

<table>
<thead>
<tr>
<th></th>
<th>Female C57 (n=20)</th>
<th>Female het (n=30)</th>
<th>Female homo (n=30)</th>
<th>Male C57 (n=37)</th>
<th>Male het (n=30)</th>
<th>Male homo (n=30)</th>
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<tbody>
<tr>
<td>total cholesterol</td>
<td>90±3</td>
<td>139±3 *</td>
<td>216±4 **#</td>
<td>101±4</td>
<td>211±4 *</td>
<td>357±19 **#</td>
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<tr>
<td>HDL cholesterol</td>
<td>71±3</td>
<td>111±2 *</td>
<td>167±4 **#</td>
<td>74±3</td>
<td>165±3 *</td>
<td>240±5 **#</td>
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<tr>
<td>triglycerides</td>
<td>57±10</td>
<td>97±6 *</td>
<td>156±16 **#</td>
<td>59±5</td>
<td>160±13 *</td>
<td>345±24 **#</td>
</tr>
<tr>
<td>FFA</td>
<td>54±5</td>
<td>73±2 *</td>
<td>82±4 **#</td>
<td>56±2</td>
<td>83±6 *</td>
<td>107±5 **#</td>
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<tr>
<td>glucose</td>
<td>95±6</td>
<td>105±9</td>
<td>106±8</td>
<td>101±7</td>
<td>111±9</td>
<td>139±11 *</td>
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<tr>
<td>insulin</td>
<td>453±105</td>
<td>522±142</td>
<td>1018±397 **#</td>
<td>537±98</td>
<td>1090±386 *</td>
<td>1752±476 **#</td>
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<tr>
<td>apoAII</td>
<td>15±1</td>
<td>39±3 *</td>
<td>66±5 **#</td>
<td>17±2</td>
<td>55±4 *</td>
<td>97±7 **#</td>
</tr>
</tbody>
</table>

* indicates values that are significantly different (p<0.05) from C57BL/6 mice of the same sex.  # indicates values that are significantly different (p<0.05) from heterozygous mice of the same sex.
Table 2. Treatment with rosiglitazone ameliorates insulin resistance in the apoAII transgenic mice. Fasting plasma lipids, glucose, insulin, and apoAI and apoAII concentrations were determined in a group of 14 apoAII transgenic mice before (Pre-treatment) and after (Post-treatment) administration of rosiglitazone in the diet (4mg/kg) for one week.

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment (n=14)</th>
<th>Post-treatment (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>total cholesterol (mg/dl)</td>
<td>252±20</td>
<td>216±17</td>
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<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>176±12</td>
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<tr>
<td>triglycerides (mg/dl)</td>
<td>212±28</td>
<td>98±14 *</td>
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<tr>
<td>FFA (mg/dl)</td>
<td>41±2</td>
<td>32±2 *</td>
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<tr>
<td>glucose (mg/dl)</td>
<td>142±9</td>
<td>109±8 *</td>
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<tr>
<td>insulin (pg/ml)</td>
<td>1880±318</td>
<td>718±110 *</td>
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<tr>
<td>apoAII (mg/dl)</td>
<td>93±8</td>
<td>85±7</td>
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<tr>
<td>apoAI (mg/dl)</td>
<td>140±13</td>
<td>127±8</td>
</tr>
</tbody>
</table>

* indicates values that were significantly different from pre-treatment values, p<0.05.
Figure 1.

![Glycogen levels in liver and muscle](image)
Figure 2.
Figure 3.

Percent expression (AIHtg versus controls)

- MCAD: p = 0.017
- H-FABP: p = 0.13
- FATP: p = 0.023
Figure 4.
Figure 5.
Figure 7.
Figure 8.

**Glucose**

- CD36 wt
  - p<0.01
  - r=0.413

- CD36 lo
  - p<0.0029
  - r=0.617

**Insulin**

- CD36 wt
  - p<0.0047
  - r=0.449

- CD36 lo
  - p<0.0146
  - r=0.525
Figure 9.