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14. ABSTRACT: There is an epidemic of obesity in the military. Obesity leads to insulin resistance syndromes, notably metabolic syndrome and type 2 diabetes. The major cause of death in these syndromes is atherothrombotic vascular disease, including coronary artery disease. Therefore, when retired military personnel and their families reach middle age, there will be an epidemic of obesity-related vascular disease. The studies completed as part of this project have revealed new cellular and molecular links between obesity/insulin resistance and atherothrombotic vascular disease. We have shown that a side-effect of a class of insulin-sensitizing PPARγ-activating drugs called TZDs, which are used widely in obese people, may promote vascular disease. We have also shown how adiponectin, a hormone that is decreased in obese subjects, might protect against vascular disease. These advances suggest new ideas for drug therapy: a "super" TZD that would eliminate the potential pro-atherogenic effects; and an adiponectin mimetic, which would protect macrophages from advanced lesional apoptosis. If successful, these drugs would help stem the rising tide of obesity-related heart disease in middle-aged and older military personnel and their families as well as in the general population.

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INTRODUCTION:

As described in detail in the original grant application, there is an epidemic of obesity in the military. Obesity leads to insulin resistance syndromes, notably metabolic syndrome and type 2 diabetes. The major cause of death in people with insulin resistance syndromes is atherothrombotic vascular disease, including acute myocardial infarction, sudden death, and stroke. Therefore, when retired military personnel and their families reach middle age, there will be an epidemic of obesity-related vascular disease. This will result in the loss of senior personnel and the expertise they contribute to the military. Moreover, the economic burden of cardiovascular disease in active and retired personnel and their families on the military will be enormous. The impact of this trend is being felt now in the military but will accelerate to a very high level over the next 10-20 years if the current trends are left unchecked. In this context, the overall objective of the proposal is to understand at a cellular and molecular level how obesity/insulin resistance promotes atherothrombotic vascular disease. Accomplishing this goal will suggest new targets for drug therapy, which would greatly benefit both the military and the general population. The emphasis of the work is on a key event in advanced atherosclerosis that leads to acute vascular events, namely, advanced lesional macrophage death. Macrophages are the major cell type in atherosclerotic lesions, and when they die and the cell corpses are not rapidly cleared by neighboring phagocytes, necrosis ensues. Plaque necrosis, in turn, promotes plaque disruption and exposure of thrombogenic material. The newly exposed thrombogenic material triggers platelet aggregation (thrombosis), which can acutely obstruct the arterial lumen and cause tissue death (infarction). In the heart, this series of events leads to myocardial infarction and sudden death, and in the brain the consequence is stroke. Thus, the overall focus of this proposal is advanced lesional macrophage death, and we have excellent progress in the first year of funding in understanding how obesity/insulin resistance can promote this event.

BODY:

Due to extremely exciting experimental data obtained early in the first year of the project, and the promise for translation into therapy that could benefit the military and general population, we have focused our efforts over the last year on items related to **Tasks 3-6**. In particular, we have explored the effects of PPARγ and adiponectin on macrophage death pathways relevant to advanced atherosclerosis. In our scientific judgment, completion of these studies, which will occur in 2007, will enable us to more intelligently address how angiotensin II affects these pathways (**Tasks 1-2 and portions of Tasks 3-4)**.

I. PPARγ studies (related to Tasks 3-6)

A. Introduction—One of the most widely used class of drugs for insulin resistance syndromes associated with obesity are thiazolidinediones (TZDs). Examples are pioglitazone (Actos) and rosiglitazone (Avandia). It is likely that these two drugs are widely used in the middle-age and older population of the military and their families, and

the use of TZDs in this population of military personnel will almost certainly grow exponentially over the next 10 years if current obesity/type 2 diabetes trends continue. These drugs were developed as PPARγ agonists, but it is well known that they have PPARγ-independent effects as well. Because atherothrombotic vascular disease, notably coronary artery disease, is the major cause of death in type 2 diabetes, there is great interest in how these drugs affect the atherothrombosis process. To the extent that TZDs improve insulin resistance, they should certainly have some degree of benefit for heart disease, as described in the original grant application. Indeed, this appears to be the case, but the magnitude of the effect in a recent large clinical trial was somewhat disappointing (1). The major questions we have been addressing through the Tasks of this proposal, spawned by our initial work with angiotensin 2, is whether TZDs have direct, arterial-wall effects on atherothrombosis independent of their effects on insulin resistance. The results of the study described below show that TZDs, and the widely used pioglitazone (Actos®) in particular, enhance macrophage death *in vitro*. Most importantly, using a well-established mouse model of advanced atherosclerosis, we found that pioglitazone promotes macrophage death and plaque vulnerability *in vivo*. These exciting findings suggest that TZDs have both beneficial effects (improving insulin resistance) and detrimental effects (promoting advanced lesional macrophage death). Our elucidation of the detrimental effects may provide an opportunity to make a better drug, *i.e.*, one with only the beneficial effects. This is particularly promising, because the detrimental effects on macrophage death appear to be PPARγ-independent. Thus, if we could eliminate the detrimental PPARγ-independent effects, we may be able to create a "super TZD." We hope this work will attract the attention of the pharmaceutical industry, leading to the creation of a better drug to treat obesity/diabetes-related cardiovascular disease.

B. Experimental work

1. Abstract—Thiazolidinediones (TZDs) inhibit early foam-cell atherogenesis in mice; however, their effects on advanced lesion progression are unknown. A unique process in advanced atherosclerosis is the accumulation of free cholesterol (FC). FC accumulation *in vivo* is associated with increased macrophage death, which in the absence of phagocytic clearance, leads to lesion necrosis and plaque instability. Because late lesional macrophages express PPARγ, we sought to determine how PPARγ agonists affect two important parameters of advanced lesion development, namely proatherogenic FC-induced macrophage death and anti-atherogenic phagocytic clearance of apoptotic macrophages. We found that micromolar concentrations of the two currently prescribed TZDs, pioglitazone and rosiglitazone, enhanced both death and phagocytosis of FC-loaded macrophages. Death enhancement was associated with reduced activation of the cell survival mediator, NFκB. Importantly, when the NFκB pathway was inhibited in FC-loaded macrophages, enhancement of death by pioglitazone was markedly decreased. PPARγ-deficient macrophages were still susceptible to increased FC-death by pioglitazone, indicating a PPARγ-independent mechanism. To determine the net direct effect of a TZD during advanced atherosclerosis, pioglitazone was administered to *Ldlr*-/- mice following previous diet-induced atherogenesis. Independent of changes in plasma glycemic control and lipid levels, plaques from pioglitazone-treated mice were more necrotic and displayed

increased TUNEL-positive cells in macrophage-rich vascular intima. Thus, in advanced atherosclerotic lesion development, potentially detrimental PPARγ-independent effects of TZDs may partially counteract their beneficial effects. These findings suggest a strategy to create PPARγ activators with a more potent anti-atherothrombotic profile.

2. Methods

a. Materials—Low-density lipoprotein (LDL; *d* 1.020-1.063 g/ml) was isolated by preparative ultracentrifugation in sodium bromide from fresh human plasma. Plasma was obtained from the New York Blood Center, New York, NY. LDL was dialyzed in 150 mM NaCl, 1mM EDTA, pH 7.4, filtered through a 0.45 um filter, and stored under argon. Protein concentration was determined by Lowry (2). LDL was subsequently acetylated with acetic anhydride (3). Compound 58-0035 (3-[decyldimethylsilyl]-*N*-[2-(4-methylphenyl)-1-phenylethyl] propanamide), an inhibitor of acyl-CoA:cholesterol *O*-acyltransferase (ACAT) (4), was used at 10 μg/ml and was provided by Dr. John Heider, formerly of Sandoz, Inc. (East Hanover, NJ). Pioglitazone hydrochloride was obtained from Sigma (product # P4120) & Alexis Biochemicals. Rosiglitazone (potassium salt) was from Cayman Chemicals. PPAR delta agonist GW501516 was from Alexis Biochemicals. Fenofibrate was obtained from Sigma. Staurosporine in DMSO (S-4400-Sigma) was used at 100 nM for apoptosis induction. PS1145 was a generous gift from Robert Schwabe. Cells were cultured on Corning tissue-culture-treated plastic and incubated in L-cell conditioned medium with heat-inactivated fetal bovine serum (Gibco).

b. Mice for eliciting and culturing of peritoneal macrophages—Macrophages were obtained from 8-10 week old female C57BL6/J mice from Jackson Laboratories. Peritoneal macrophages were harvested after an immunization protocol using intradermal and intraperitoneal methyl-BSA or after eliciting with intraperitoneal concanavalin A or thioglycollate as indicated. Macrophages were harvested with cold phosphate buffered saline by peritoneal lavage. Cells were spun down, suspended in L-cell conditioned medium, and filtered. Macrophages were selected by adherence to tissue culture-treated plastic and cells were incubated in DMEM with 10% FBS & 20% L-cell-conditioned medium and medium replaced every 24 hours until 80% confluency. Macrophages were cultured for two to three days prior to cholesterol loadings. PPARγ-deficient mice were generously provided by Frank J. Gonzalez and Yatrik Shah. These mice were generated using the LysMCre-loxP system.

c. Cholesterol loading and apoptosis measurements—Macrophages were rinsed in PBS and incubated with 100 μg ml^{-1} acetyl-LDL plus 10 μg/mL 58035 (to inhibit ACAT-mediated cholesterol esterification and thus induced FC accumulation). Early to mid-stage apoptosis (externalization of phosphatidylserine) was measured by addition of Alexa-488-labelled annexin, and late-stage apoptosis was assessed by staining with PI, an indicator of increased membrane permeability. Micrographs were captured with an Olympus IX-70 inverted fluorescence microscope and 5 representative fields (approximately 1,000 cells) for each condition were counted for the number of annexin-positive, PI-positive, and total cells.

d. Acetyl-LDL degradation and whole-cell cholesterol esterification measurements—For CE measurements, primary macrophages were incubated for 5

hours in DMEM 0.2% BSA containing 50 μg/mL [^{14}C]oleate-labeled acetyl-LDL alone or in the presence of pioglitazone. Cellular lipids were extracted twice with 0.5 mL of hexane:isopropanol (3:2 v:v) and cellular [^{14}C]cholesteryl ester determined by thin-layer chromatography. The lipid-extracted cell monolayer was dissolved in 1 N NaOH and assayed for protein content via Lowry.

e. Subcellular fractionations and immunoblots—Nuclear extractions per Panomics. Approximately 10 μg total protein loaded per 15-well minigel and electrophoresis on a 4-15% Tris-HCL gradient gel. Proteins on nitrocellulose detected via ECL.

f. Phagocytosis—Efferocytosis was assessed as previously described with some modifications as described below (5). Primary peritoneal macrophage phagocytes were selected via adherence to tissue culture plastic and incubated in L-cell conditioned medium for two to three days. In some cases, phagocytes were fluorescently labeled prior to loading apoptotic cells. Apoptotic cells were derived from either FC-loading of methyl-BSA-elicited peritoneal macrophages or after UV-irradiation of J774 cells as indicated. Prior to inducing apoptosis, cells were fluorescently labeled with calcein AM (Molecular Probes). For UV-induced apoptosis, adherent J774 cells were subjected to irradiation for 15 minutes at 254nm, 20 J/cm^2. Early apoptotic cells were loaded after morphological manifestation of membrane blebs. This was confirmed in parallel assays via annexin V and propidium iodide (PI) staining. In the case of FC-loading, typically 20-30% of cells were apoptotic per annexin V staining and less than 5% necrotic per PI staining. Apoptotic cells were harvested, pelleted by centrifugation, suspended in serum-containing DMEM, and overlaid onto adherent macrophages at a 1:1 ratio. After 30-45 minutes, non-ingested and adherent cells were removed after vigorous monolayer agitation and rinsing. Cells were fixed in paraformaldehyde and phagocytic uptake enumerated. To confirm uptake, monolayers were inspected by confocal microscopy.

g. Feeding Protocols—Male and female *Ldlr*-/- mice on a C57BL/6J background (B6.129S7-Ldlrtm1Her, stock # 002207) were purchased from The Jackson Laboratories (Bar Harbor, ME, USA) and bred in an animal barrier facility at Columbia University Medical Center, New York, NY. Mice were weaned at 4 weeks and five animals were housed per cage under an 12-hour light cycle. All animal protocols were approved by IACUC. Diet-induced hypercholesterolemia was started at 6 weeks of age. Mice were fed a gamma-irradiated low-fat (10 kcal fat), high cholesterol (0.5% or 5.3 gm cholesterol/4057 kcal) semi-synthetic (AIN76) diet from Research Diets (NJ) for 8 weeks at ~3 grams per day (6). Subsequently, mice were split into two groups, one of which received pioglitazone in addition to the semi-synthetic diet. According to food intake, the dose of pioglitazone was 40 mg/kg of body weight/day, in addition to the semi-synthetic diet. Pioglitazone was administered for 18 weeks, for a total high cholesterol diet duration of 18 weeks). Alternatively, pioglitazone was give at the start of the semi-synthetic diet to monitor effects on early lesion development.

h. Blood Chemistries/Plasma Analysis—Plasma was collected after a 12 hour fast and stored at –70^{o}C. Glycemic and lipid parameters were measured enzymatically. Total plasma cholesterol was measured with Wako's cholesterol oxidase kit. HDL was measured by phosphotungstate-magnesium salt precipitation per Wako's

HDL-Cholesterol E kit code No. 431-52501. Fasting plasma glucose was measured enzymatically using Biovison's Glucose Assay Kit (catalog #K606-100). Cholesterol distribution within lipoproteins was determined by separating lipoproteins by fast performance liquid chromatography (FPLC) gel filtration on a Superose 6 column at a flow rate of 0.2 mL per minute.

i. Vascular tissue preparation and morphometric lesion analysis— Mice were weighed and anesthetized. The heart was removed by cutting the ascending aorta midway between the aortic root and brachiocephalic artery and subsequently fixed. Paraffin-embedded sections were cut at 6-μm intervals.

j. Immunohistochemistry and in situ TUNEL analysis—Paraffin sections were deparaffinized in xylene and rehydrated in graded series of ethanol. Antigens were retrieved via heating in an EDTA solution followed by hydrogen peroxide/methanol blocking of endogenous peroxidase. Blocking was performed using immunoglobulin from the species of the secondary antibody. For SMC staining of primary mouse antibody on mouse tissue, we followed the protocol of Zymed's Histomouse^{TM}-SP Kit. Apoptotic cells in atherosclerotic lesions were detected by TUNEL (Tdt-mediated dUTP nice end labeling) using the in situ cell death detection kit, TMR red from Roche. Sections of proximal aorta were deparaffinized and treated with proteinase K followed by initiating the TdT reaction. Nuclei were counterstained with Hoechst for 5 minutes and slides examined by fluorescent microscopy. Images merged and quantified with Adobe Photoshop.

k. Statistics—Data are presented as mean +/- S.E.M.

3. Results

a. TZDs elevate free cholesterol (FC)-induced macrophage apoptosis independently of PPARγ—To determine the effects of PPARγ ligands on macrophage viability in the context of advanced atherosclerosis, we added pioglitazone to FC-loaded primary macrophages in tissue culture and stained for apoptosis using Annexin V. FC-loading was induced by incubating primary macrophages with acetylated LDL and ACAT inhibitor 58-035. Pioglitazone-treated macrophages, on average, exhibited an approximate 2-fold increase in susceptibility to FC-induced death at 10 μM (**Fig. 1A**). In contrast, the effect of pioglitazone on death of cholesteryl ester-loaded macrophages (foam cells) and unloaded cells, which predominate in early lesions, was minimal (**Fig. 1B**). Death enhancement by pioglitazone followed a direct dose-response relationship, with elevations in apoptosis measured as low as 100 nM (**Fig. 1C**). Furthermore, FC-induced apoptosis was recapitulated with another TZD, rosiglitazone (**Fig. 1D**). Finally, elevated FC-induced apoptosis was specific to PPARγ ligands, as the PPARα ligand fenofibrate and the PPARδ ligand GW501516, did not increase Annexin V staining at 1 μM (data not shown).

Previous work from our laboratory has shown that accumulation of lipoprotein-derived FC in the endoplasmic reticulum (ER) induces the unfolded protein response (UPR), which is required for FC-induced macrophage apoptosis (7). To determine whether enhancement of FC-induced apoptosis was the result of increased cellular cholesterol internalization or elevated intracellular cholesterol trafficking to the ER, we

measured [^{125}I]acetyl-LDL degradation and cholesteryl esterification respectively. We found that pioglitazone did not increase either of these processes and, consistent with this finding, did not increase the expression of the UPR death effector CHOP (data not shown). Instead, apoptosis enhancement was a more general phenomenon, as shown by the increase in apoptosis by pioglitazone in macrophages treated with either the ER-stressor thapsigargin (**Fig. 2A**) or the nonselective protein kinase inhibitor, staurosporine (**Fig 2B**).

The effects of TZDs do not always correlate with levels of PPARg expression (8). To determine whether elevated apoptosis required PPARγ, we studied primary peritoneal macrophages from PPARγ^{flox} mice or PPARγ^{flox} x LysMCre mice. As shown in **Fig. 3B**, LysMCre expression was effective in knocking down PPARγ expression. The two groups of macrophages were loaded with FC in the absence or presence of pioglitazone, and the degree of apoptosis was assessed. As shown in **Fig. 3A**, PPARγ-deficient macrophages remained susceptible to pioglitazone-mediated death enhancement. These data indicate that the enhancement of apoptosis by pioglitazone does not involve activation of PPARγ.

b. Pioglitazone reduced activity of pro-survival NFkB in ER-stressed macrophages—We previously reported that FC-loading triggers the NFκB pathway in macrophages (9). NFκB activation can drive pro-survival responses in many cell types (10). Moreover, pioglitazone has been shown to antagonize the NFκB pathway *in vivo* (11). We therefore hypothesized that pioglitazone enhanced FC-apoptosis through inhibition of NFκB activity in FC-loaded macrophages. In support of this hypothesis, death enhancement was associated with reduced nuclear translocation of NFκB p65 (**Fig. 4A**). This was not due to reductions in total cellular p65, as we measured no difference in whole-cell extracts (data not shown). Importantly, when the NFκB pathway was inhibited in FC-loaded cells with the IKK inhibitor PS1145, enhancement of death by pioglitazone was markedly decreased (**Fig. 4B**), implicating NFkB activation as a pro-survival response in FC-loaded cells.

c. Pioglitazone & rosiglitazone enhance phagocytic uptake of free cholesterol-loaded apoptotic macrophages (FC-AMs)—The consequences of macrophage apoptosis in atherosclerotic lesions are directly linked to the efficiency of efferocytosis (phagocytic clearance of apoptotic cells) (12). Inefficient clearance permits post-apoptotic necrosis and unregulated liberation of pro-inflammatory mediators. To determine the effects of TZDs on efferocytosis, we added pioglitazone and rosiglitazone to macrophage phagocytes. Subsequently, fluorescent FC-AMs were added, and phagocytic uptake quantified by microscopy. As shown in **Fig. 5A-B**, phagocytes treated with the TZD rosiglitazone ingested significantly more FC-AMs than untreated phagocytes. TZD enhancement of phagocytosis was specific to apoptotic cells as we found no effect on uptake of immunoglobulin-opsonized sheep erythrocytes. We next tested the role of PPARγ on efferocytosis of FC-AMs. As shown in **Fig. 5C**, PPARγ deficiency led to enhanced FC-AM uptake even in the absence of TZD treatment (compare 1st and 3rd bars). Furthermore, TZDs failed to promote increased phagocytosis above the level of this enhanced basal uptake (compare 3rd and 4th bars). These data are consistent with two processes: a dominant suppressive effect of basal

PPARγ activation and an enhancing effect of rosiglitazone by a mechanism that is yet to be elucidated.

d. Advanced lesions from pioglitazone-treated mice are more necrotic—In the context of our *in-vitro* findings, we sought to determine the net effect of pioglitazone treatment on advanced lesion development. To study the direct vascular effects of pioglitazone during advanced lesions progression, *Ldlr*-/- mice were fed a semi-synthetic low-fat, high cholesterol diet for 8 weeks to generate intermediate to advanced lesions (6). Subsequently, the semi-synthetic diet was continued for 10 weeks in the presence or absence of pioglitazone. At the end of the protocol, plasma metabolic/lipid parameters were assessed, and aortic root lesions were analyzed morphometrically. As shown by the table in **Fig. 6A**, mice were mildly hyperinsulinemic but euglycemic. Although insulin levels trended lower in the pioglitazone-treated group, there was no statistical difference between control and drug-treated mice with respect to glucose and insulin levels. Pioglitazone reduced total fasting plasma cholesterol by ~10% and increased HDL-cholesterol by ~25%. Chromatograms of plasma lipoproteins from pioglitazone-treated mice showed mild cholesterol reductions in the VLDL and LDL fractions (**Fig. 6B**).

As shown in **Fig. 7**, there was no difference in total aortic root lesion area between the two groups of mice. However, plaques from the pioglitazone-group displayed more pronounced necrotic cores and increased acellular area, an indicator of cellular death and predictor of plaque vulnerability. To investigate whether increased lesion acellularity in the pioglitazone-treated group was associated with increased lesion macrophage apoptosis, we performed TUNEL analysis on proximal aorta cross sections. Sections from the pioglitazone group had increased nuclear-specific TUNEL staining (**Fig. 8**). TUNEL-positive regions stained positively for macrophages and not smooth muscle cells, suggesting the presence of apoptotic macrophages.

4. Discussion—This study sought to examine the direct vascular effects of TZDs ligands during advanced lesion progression. Though clinically silent, advanced lesions are the critical stage of human atherosclerosis. They are the precursor to plaque rupture and acute thrombotic vascular occlusion. Here we show that pioglitazone, when administered to mice with pre-established lesions, can exacerbate important precursors to plaque rupture, namely, expansion of lesional macrophage death and plaque necrosis. This consequence occurred even in the face of improved lipid and glycemic parameters. Plaque size however, was not reduced, similar to a recent study using the same dose of pioglitazone (13).

In vitro, pioglitazone-mediated elevations in apoptosis were pronounced in FC-loaded cells but not in non-apoptotic unloaded or cholesteryl-ester loaded cells, which are the types of cells that predominate in early lesions. Thus, TZDs act as apoptosis enhancers, not apoptosis inducers. Consistent with this concept, we failed to measure increased macrophage apoptosis in lesions from a small group (n=6) of mice treated with pioglitazone during early lesion development. Thus, TZD-mediated increases in macrophage apoptosis are likely specific to advanced lesion development.

Previous studies have highlighted the capacity of PPARγ ligands to promote apoptosis in various vascular cell types. For example, TZDs increase vascular smooth muscle cell apoptosis in tissue culture and have further been shown to induce regression of intimal hyperplasia in balloon-injured rat carotid arteries, concomitant with increases in neointimal cell apoptosis (14,15). Regarding macrophages, pioglitazone was shown to increase $CD68^+$ TUNEL staining and reduce macrophage content n white adipose tissue from type II diabetics (16). Chinetti *et al.* (17) reported that TZDs increase apoptosis of non-activated differentiated macrophages *in vitro* concomitant with reductions in NFκB activation. This enhancement could be recapitulated with PPARα ligands and was more pronounced after cultivating with TNFα and IFNγ. In our hands, we did not observe increased FC-induced apoptosis with either PPARα or PPARδ ligands (data not shown).

An important cell-survival modulator in the context of FC-loading is NFκB. Previously we have shown that ER-stressed macrophages trigger NFκB nuclear translocation and downstream proinflammatory mediators (9). Activation of NFκB has been reported to antagonize programmed cell death in many cell types (18). In line with its pro-survival role, inhibition of IKK, the upstream activator of NFkB, enhanced FC-apoptosis (**Fig. 4**). TZDs have been shown previously to inhibit NFκB activity by either PPARg-dependent or –independent mechanisms (19-21). Our findings would suggest PPARγ-independent inhibition of NFκB activity is a key mechanism of apoptosis enhancement. Of interest this regard, while the inflammatory aspects of NFκB are thought to be pro-atherogenic, the net effect of NFκB inhibition is exacerbation of lesion development (22).

Pioglitazone (Actos®) and rosiglitazone (Avandia®) are widely prescribed oral insulin-sensitizing agents in type II diabetics. One would presume that their ability to ameliorate insulin resistance would have an overall beneficial effect in acute cardiovascular events in type II diabetics. Indeed, a trend toward this result was found in the PROactive trial (1), but the magnitude of the protective effect was less than expected (23). The findings in this report suggest the possibility that the beneficial effects of TZDs, notably those on systemic metabolic parameters, may be counterbalanced by direct arterial wall effects that are detrimental. To the extent that the biological mechanism of the effect elucidated here was PPAR independent, future drugs that have more specificity for PPARγ activation may have more beneficial effects on macrovascular disease.

5. Figure legends:

Figure 1. PPARγ ligands increase FC-induced macrophage apoptosis. *A*, Macrophages were cultured in either DMSO or 10 μM pioglitazone (PIO) for 18-24 h. The cells were then incubated with medium ± 50 μg/mL of acetyl-LDL plus the ACAT inhibitor 58035 (FC), also in the absence or presence of pioglitazone. Twelve hours later, the cells were stained with annexin V and propidium iodide and viewed by fluorescence microscopy. ***B*,** Quantification of apoptosis data, which also includes cells incubated with acetyl-LDL alone to effect non-apoptotic cholesteryl ester loading (CE). $p < 0.05$ for FC *vs*. FC-PIO. ***C*,** Macrophages were pretreated with the indicated micromolar doses of pioglitazone before and during FC loading as described in A and

then quantified for percent apoptosis. ***D*,** Macrophages were pretreated in the absence or presence of 10 μM pioglitazone or 10 μM rosiglitazone (rosi) before and during FC-loading as described in A and then quantified for percent apoptosis. *, significantly different from untreated cells.

Figure 2. Pioglitazone is a general enhancer of macrophage death. Quantification of annexin V and PI staining of macrophages after incubation in medium containing either **(*A*)** thapsigargin or **(*B*)** staurosporine. Pioglitazone was added 24 h prior to and throughout thapsigargin or staurosporine treatment.

Figure 3. Pioglitazone increases FC-apoptosis in PPARγ-deficient macrophages. ***A*,** Macrophages from $PPAR\gamma^{flox}$ (fl/fl) or $PPAR\gamma^{flox}$ x LysMCre (fl/fl + Cre) mice were FC-loaded ± pioglitazone (+P) and assayed for apoptosis as in the previous figures. Each of the plus-pioglitazone values (black bars) are statistically different ($p < 0.05$) from the respective no-pioglitazone values (white bars) for each group of macrophages. ***B*,** Immunoblot analysis of PPARγ in macrophage nuclear extracts for the four conditions investigated in A.

Figure 4. Pioglitazone reduces NFκB nuclear translocation in FC-loaded macrophages. ***A*,** Primary macrophages were loaded with AcLDL and 58035 ± 10 μM pioglitazone. At the indicated hours post FC-loading, nuclear extracts were prepared and subjected to immunoblot for nuclear p65. Blots were stripped and reprobed for nucleophosmin as a nuclear loading control. ***B*,** Free-cholesterol loaded macrophages were incubated in the presence of DMSO vehicle, 10 μM pioglitazone, or 10 μM PS1145 as indicated. Approximately 11 h post FC-loading, cell monolayers were stained for apoptosis via Annexin V staining. ** indicates $p < 0.05$ relative to DMSO control.

Figure 5. TZDs enhance efferocytosis of FC-induced apoptotic macrophages. ***A*,** Macrophages were labeled with the fluorophore calcein-AM (green) and loaded with FC to induce apoptosis (FC-AMs). Green FC-AMs were overlaid onto octadecylrhodamine-labeled (red) phagocytes for 30 min at 37°C. These phagocytes were pre-incubated for 18 h with 1 μM rosiglitazone or DMSO vehicle control. ***B*,** Quantified data for FC-AM uptake by phagocytes pre-treated with pioglitazone (P) or rosiglitazone (R). ***C*,** Quantified data for FC-AM uptake by phagocytes $PPAR\gamma^{flox}$ (fl/fl) or $PPAR\gamma^{flox}$ x LysMCre (Cre) mice.

Figure 6. Metabolic and lipid profile of *Ldlr*$^{-/-}$ mice after being fed a high-cholesterol diet ± pioglitazone. *Ldlr*-/- mice were fed a high-cholesterol diet and then treated with or without pioglitazone as described under Methods (above). ***A*,** Plasma samples were assayed for glycemic and lipid parameters as indicated. ***B*,** Pooled plasma samples were subjected to fast performance liquid chromatography gel-filtration fractionation and assayed for cholesterol.

Figure 7. Quantitative characterization of advanced plaques from control and pioglitazone-treated *Ldlr-/-* mice. Adjacent serial sections of proximal aortic lesions from cholesterol-fed *Ldlr*-/- mice treated with or without pioglitazone were stained with hematoxylin and quantified for lesion area and lesion acellular area, as described under Methods.

Figure 8. TUNEL staining & immunohistochemistry of proximal aortae from control and pioglitazone-treated *Ldlr-/-* mice. Atherosclerotic plaques in the proximal aortae of control and pioglitazone-treated mice were assayed for TUNEL positivity (Tdt), Hoechst (nuclei), macrophages (Mϕ), and vascular smooth muscle cells (VSMC) as described under Methods. Examples of representative sections from 2 mice as well as quantitative data from the full group of mice are shown.

6. Figures:

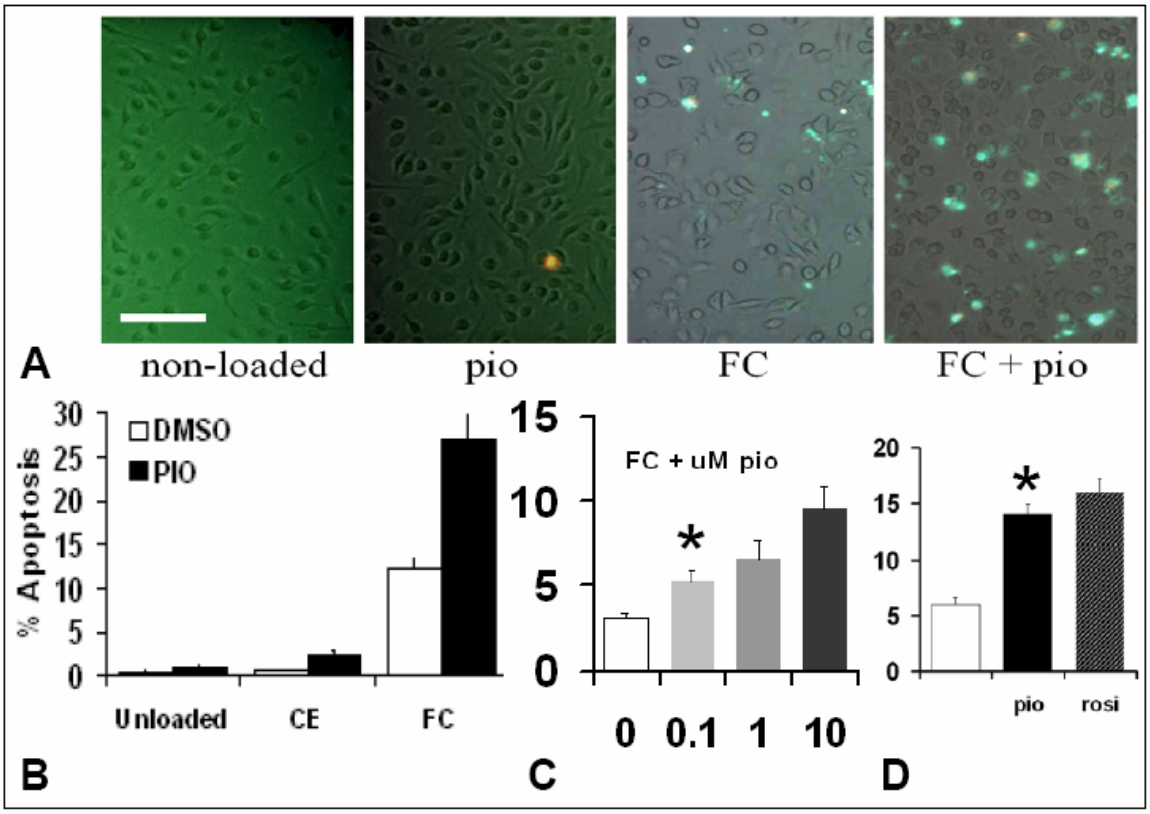


Figure 1

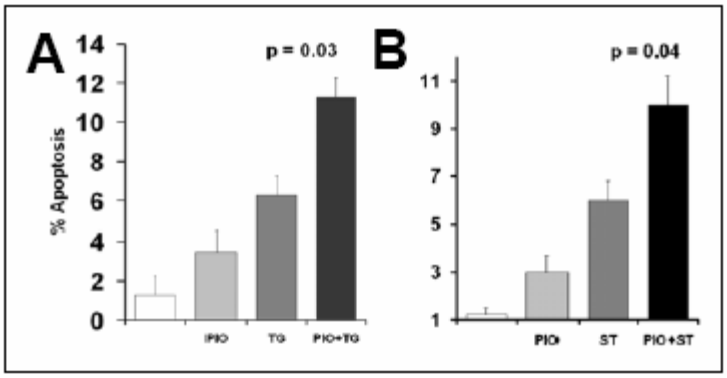
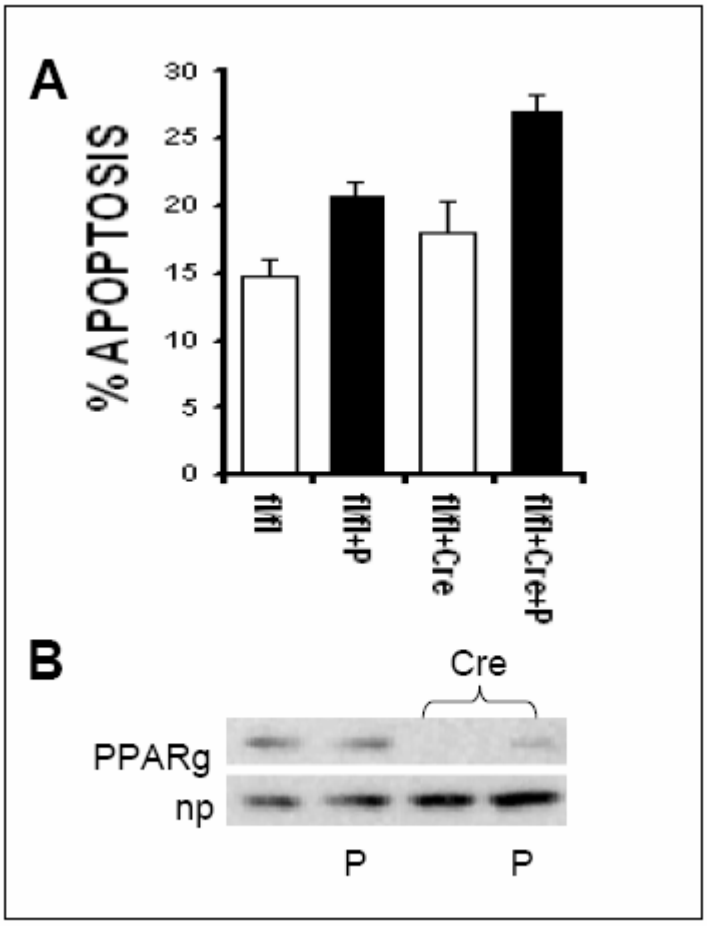


Figure 2



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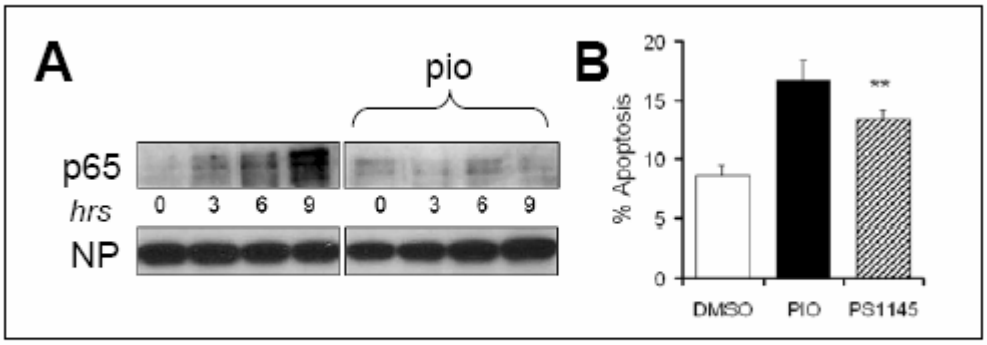

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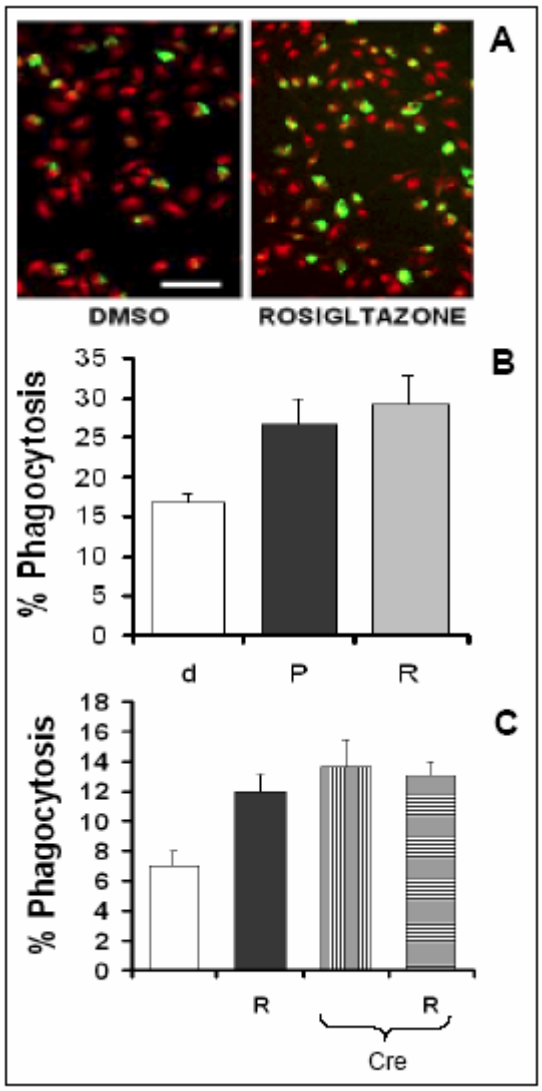


Figure 5

A

	control	pioglitazone	p
Body Weight (g)	32.7 ± 0.5	32.9 ± 0.8	0.78
Glucose (mg/dl)	76.1 ± 2.5	81.2 ± 2.0	0.12
Insulin (pg/ml)	1,513.6 ± 106.4	1,335 ± 131.5	0.09
Total Cholesterol (mg/dl)	1,138.2 ± 33.8	949.8 ± 38.3	<0.01
HDL-Cholesterol (mg/dl)	64.5 ± 3.9	90.7 ± 4.5	<0.01
Triglycerides (mg/dl)	130.4 ± 12.7	109.7 ± 15.9	0.32

B

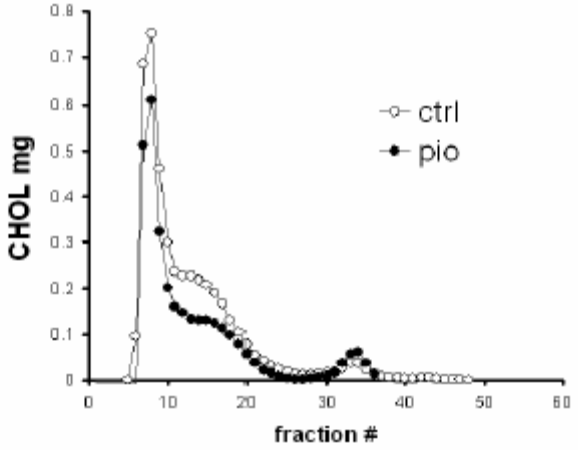


Figure 6

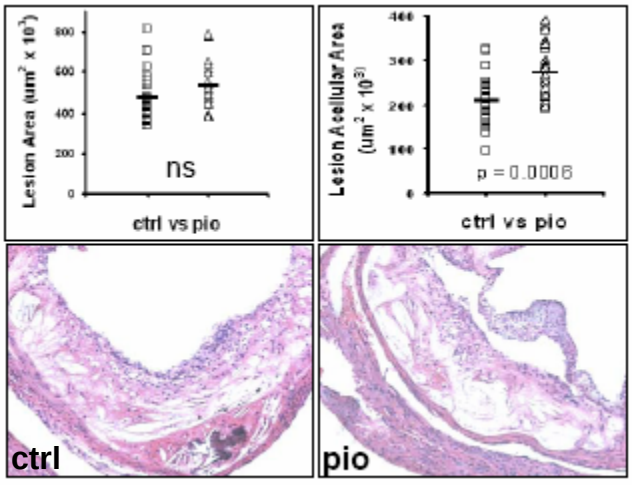


Figure 7

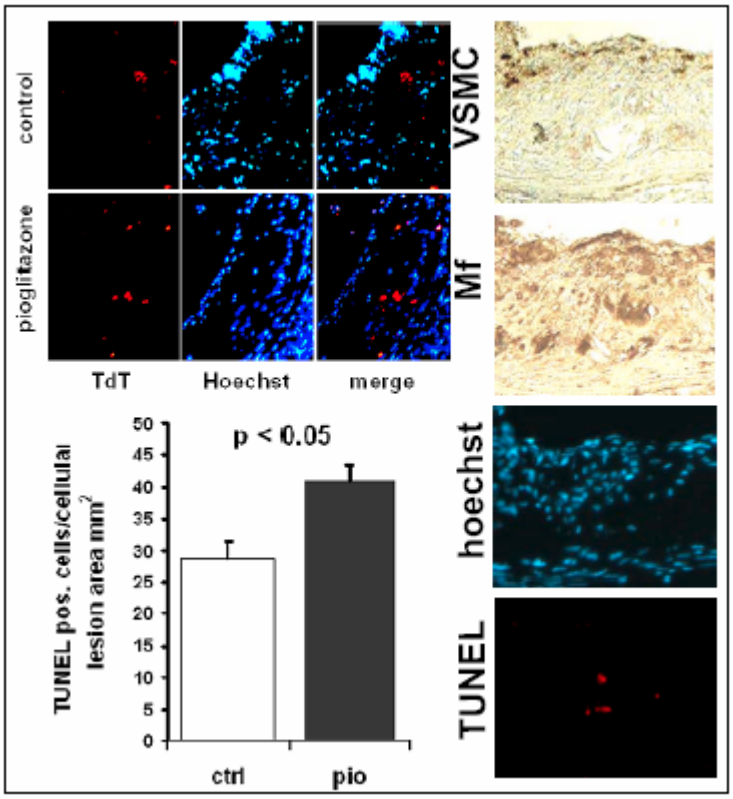

control
pioglitazone
TdT
Hoechst
merge
VSMC
Mf
hoechst
TUNEL
p < 0.05
TUNEL pos. cells/cellular lesion area mm²
0
5
10
15
20
25
30
35
40
45
50
ctrl
pio

Figure 8

II. Adiponectin studies (related to Task 7)

A. Introduction—In thin individuals, fat cells make an abundant circulating protein called adiponectin. This protein is thought to be protective against both insulin resistance and heart disease, although the mechanisms are not well known. In obese individuals, the fat cells make much less of this protein, and so the circulating concentrations are much lower. This drop in adiponectin has been hypothesized to increase the risk of heart disease in people with obesity and insulin resistance. As described in the original grant application, we devised the specific hypothesis that the drop in adiponectin levels in obese people has a detrimental effect on advanced lesional macrophage death and plaque necrosis. Work on this Task over the last year has provided important new data on how adiponectin affects the pathways of macrophage death that are thought to be important in advanced atherosclerosis, particularly the endoplasmic reticulum stress pathway known as the Unfolded Protein Response (UPR). Understanding the cellular and molecular mechanisms of how adiponectin protects macrophages from death can lead to new drugs the mimic the protective effect of adiponectin. Such drugs would be expected to lower the risk of heart disease in the large and growing number of military and non-military personnel that are affected by obesity and obesity-related insulin resistant syndromes.

B. Experimental work

1. Abstract—Macrophages in advanced atherosclerotic lesions accumulate excess unesterified, or "free," cholesterol (FC), which activates the endoplasmic reticulum (ER) stress pathway known as the unfolded protein response (UPR) and UPR-induced apoptosis (7,24). Macrophage death in advanced atheromata leads to plaque necrosis and disruption, which are the immediate precursors of acute atherothrombotic vascular events (24). Adiponectin is an adipocyte-secreted hormone whose plasma levels are very high in lean subjects but low in obese and insulin-resistant individuals (25). In mice, adiponectin, particularly a specific multimeric form known as high-molecular-weight (HMW) adiponectin, is an insulin sensitizer (25,26). However, the molecular and cellular mechanisms are uncertain, and there have been no reports of cell-based assay or molecular targets that can distinguish HMW-adiponectin from lower molecular weight forms. Importantly, adiponectin is inversely associated with atherosclerosis in mice (25), and low plasma adiponectin is independently associated with increased risk of coronary artery disease in humans (27), but here also the mechanisms are uncertain. Here we show that in macrophages subjected to ER stress by FC loading and other means, HMW-adiponectin blocks the induction of distal UPR effectors, including apoptogenic CHOP (GADD153), and suppresses FC-induced macrophage apoptosis. The molecular mechanism of distal UPR suppression involves translational repression of a key UPR intermediate, ATF4. These findings reveal a novel molecular target of HMW-adiponectin and provides a plausible mechanism for how adiponectin might block an important step in the progression of atherothrombotic cardiovascular disease. Moreover, given the presence and functional importance of macrophages in adipose tissue, these findings may have implications related to the role of HMW-adiponectin in obesity and insulin resistance.

2. Methods

a. Production of Adiponectin. Adiponectin secreted by HEK 293T cells transfected with a bicistronic expression vector containing the cDNAs for mouse adiponectin and green fluorescent protein was purified as described previously (28).

b. Free Cholesterol loading of mouse peritoneal macrophages. Peritoneal macrophages from adult female C57BL6J mice were harvested three days after i.p. injection of concanavalin A (7), or four days after i.p. injection of methyl-BSA (mBSA) in mice previously immunized with this antigen (29). The macrophages were maintained in full medium containing DMEM, 10% FBS, and 20% L-cell conditioned medium. The medium was replaced every 24 h until cells reached 90% confluency. One the day of the experiment, the cells were washed three times in warm PBS and incubated as described in each figure legend. The cells were FC-loaded loaded by incubation with medium containing 10 μg/ml of the ACAT inhibitor 58035 plus 50 μg/ml of acetyl-LDL.

c. Macrophage apoptosis assay. Macrophages were assayed for early to mid-stage apoptosis by staining with Alexa 488-conjugated Annexin V (green), and late stage apoptosis by co-staining with propidium iodide (red), as described previously (7).

d. Immunoblot analysis of UPR proteins. Whole cell lysates were prepared in a buffer containing 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 50 mM DTT, and 0.01% bromphenol blue and boiled at 100ºC for 5 min. Cytosolic and nuclear extracts were isolated using the Nuclear Extraction Kit (PANOMIC, Redwood City, CA) according to the manufacturer's protocol. Immunoprecipitations and immunoblotting of IRE1, PERK, and GADD34 were conducted as previously described (30).

3. Results and Discussion—Because *in vivo* studies have shown that adiponectin function is influenced by post-translational modification and quaternary structure (26), the hormone was prepared using a mammalian cell transfection system that secretes the three major forms of adiponectin: HMW, low-molecular weight (LMW), and trimer. The media was then subjected to anion-exchange and size-exclusion fractionation to isolate the individual forms. To investigate a hypothesis on how adiponectin might protect against atherothrombotic vascular disease, HMW- and LMW-adiponectin were tested for their ability to affect FC-induced macrophage apoptosis. FC-loading of macrophages, modeled by incubation of the cells with acetylated low-density lipoprotein (acetyl-LDL) and the cholesterol esterification inhibitor 58035, is a potent inducer of apoptosis (7), as shown by the annexin V-staining data in **Fig. 1a**. While HMW-adiponectin itself had no significant effect on basal apoptosis in macrophages, the hormone suppressed FC-induced apoptosis to basal levels (**Fig. 1a**). Remarkably, LMW adiponectin, a form with less insulin-sensitizing activity *in vivo* (26), did not suppress FC-induced apoptosis at all (**Fig. 1b**). The trimer form of adiponectin also did not suppress apoptosis (data not shown).

Possible mechanisms of suppression of FC-induced apoptosis by HMW-adiponectin were decreases in acetyl-LDL uptake, FC mass accumulation, and/or trafficking of lipoprotein-derived cholesterol to the ER, all of which are necessary for apoptosis (7). However, the hormone had no substantial effect on acetyl-LDL uptake, FC mass

accumulation, or lipoprotein-stimulated cholesterol trafficking to the ER (data not shown). The latter was assessed by determining acetyl-LDL-stimulated cholesterol esterification by the ER-resident enzyme acyl-CoA:cholesterol acyltransferase (ACAT) in cells not receiving 58035.

Another possible mechanism was suppression of the UPR effector CHOP (GADD153), which we previously showed is induced by FC loading and necessary for the full apoptotic response in FC-loaded macrophages (7). As expected, CHOP was markedly induced by FC loading. However, in macrophages pre-treated with HMW-adiponectin, CHOP levels were suppressed in a dose-dependent manner by the hormone (**Fig. 2a**). Consistent with the apoptosis data in Fig. 1, LMW-adiponectin did not appreciably suppress FC-induced CHOP (**Fig. 2b**). To extend these findings to other UPR activators, we tested all three forms of adiponectin on UPR activation induced by tunicamycin, an inhibitor *N*-linked protein glycosylation and protein folding (31). As with FC loading, HMW-adiponectin, but not the lower molecular weight forms, suppressed CHOP (**Fig. 2c**). Similarly, HMW-adiponectin suppressed CHOP induction by the UPR activator thapsigargin (**Fig. 2d**), which inhibits sarco-endoplasmic reticulum calcium ATPase (SERCA) and thereby depletes ER calcium stores (32).

To determine where in the UPR pathway suppression by HMW-adiponectin was occurring, macrophages were pre-incubated in the absence or presence of HMW-adiponectin followed by treatment with or without tunicamycin, and then various cellular fractions were subjected to immunoblot analysis. IRE1α and PERK are upstream UPR molecules whose activation can be assessed by a subtle decrease in eletrophoretic migration secondary to phosphorylation (7,33). As expected, tunicamycin induced this change in migration for both molecules, and these effects of tunicamycin were not altered in macrophages pretreated with HMW-adiponectin (**Fig. 3a,b**). PERK phosphorylates and thus inhibits the translation initiation factor, eIF2α, which is a critical step in the UPR pathway (33). Consistent with the PERK data above, tunicamycin induced eIF2α phosphorylation, and this was also not inhibited by HMW-adiponectin (**Fig. 3c**). However, the induction by tunicamycin or FC loading of four key distal UPR effectors, ATF4, XBP-1, CHOP, and ATF3, were markedly suppressed by HMW-adiponectin (**Fig. 3c,d**). GADD34, which is another distal UPR molecule, albeit one subjected to induction by other pathways (34), was partially suppressed by HMW-adiponectin (**Fig. 3c, bottom blot**). Because GADD34 can dephosphorylate phospho-eIF2α (35), its partial suppression may explain the modest increase in phospho-eIF2α in adiponectin-treated macrophages (**Fig. 3c, top blot**).

ATF4 plays a central role in distal UPR activation, because it can transcriptionally induce ATF3, CHOP, XBP-1, GADD34, and itself (30,36,37). In non-ER-stressed cells, ATF4 protein is maintained at low levels by translational repression. The mechanism is engagement of an inhibitory open-reading frame (ORF) in the 5' untranslated region of the ATF4 mRNA that occurs in the presence of active (non-phosphorylated) eIF2α (38). Under conditions of ER stress, this inhibitory ORFs is not engaged due to partial deactivation (phosphorylation) of eIF2 . .and translational repression is relieved (38). In other conditions, post-translational control of ATF4 has been reported (39). To determine the mechanism of suppression by HMW-adiponectin, ATF4 mRNA levels were assayed in macrophages treated as above. As shown in **Fig. 4a**, tunicamycin

caused a progressive increase in ATF4 mRNA, as expected by the fact that ATF4 induces its own transcription (30). However, except for a modest and transient effect at 3 h, HMW-adiponectin did not appreciably alter ATF4 mRNA levels (**Fig. 4a**). These data indicate that adiponectin regulates ATF4 at a post-transcriptional level.

We next sought to obtain *in-vivo* data to show that the action and mechanism of adiponectin described herein is physiologically relevant. To achieve this goal, we devised an experimental assay whereby macrophage UPR activation would be influenced by endogenous circulating adiponectin *in vivo*. In this assay, mice are injected intraperitoneally with tunicamycin, a UPR inducer. The macrophages in the peritoneal cavity are exposed to the tunicamycin, and the degree of UPR activation in these macrophages is influenced by the *in-vivo* milieu the macrophages are exposed to in the peritoneum, which is richly connected to the vasculature and thus to circulating hormones like adiponectin. Thus, wild-type and adiponectin-null mice were injected with tunicamycin or vehicle control, and 5 h later, the macrophages were harvested from the peritoneum and assayed immediately for expression of phospho-eIF2α and CHOP by immunoblotting. Note that the mice in this experiment are thin, and so the wild-type mice have high circulating levels of adiponectin. As shown in **Fig. 5**, tunicamycin led to increased phospho-eIF2α and CHOP expression in the macrophages from the wild-type mice, as expected. Most importantly, CHOP expression was enhanced in the macrophages withdrawn from the tunicamycin-treated adiponectin-null mice. This finding is consistent with our data showing that adiponectin suppresses the UPR, because the wild-type mice have high levels of adiponectin and the null mice have none. Moreover, the increased CHOP expression in the macrophages from the adiponectin-null mice was accompanied by a slight decrease, not an increase, in phospho-eIF2α. This finding is extremely important, because it is consistent with the unique mechanism of action we revealed *in vitro*, namely regulation of ATF4 downstream of eIF2α (above). For example, if the mechanism *in vivo* were completely different and the increased CHOP seen in the macrophages from the null mice was an artifact of a unintentional increase in tunicamycin given to these mice, phospho-eIF2α expression would have been higher, not lower. In summary, these data show that endogenous, circulating adiponectin can suppress the UPR in macrophages *in vivo*.

The most convincing data related to the action of adiponectin involve complex *in-vivo* insulin-resistant models where improvement of hyperglycemia is the endpoint (25,26). Cell culture studies have attempted to probe mechanism, revealing possible roles for a number of cell-surface receptors and signaling intermediates (25), but *in-vivo* significance of these mechanisms and molecules is uncertain. In particular, two cell-surface receptors, AdipoR1 and AdipoR2, and the cellular energy sensor AMP-activate protein kinase (AMPK), have been the subject of intense investigation into adiponectin signaling (25). Our preliminary studies using macrophages from gene-targeted *Adipor1-/-*, *Adipor2-/-*, *Ampka1-/-*, and *Ampka2-/-* mice have suggested that none of these molecules are involved in the suppression of the distal UPR or FC-induced apoptosis by HMW-adiponectin (data not shown), but further investigation is needed. Of significance in this regard and in the context of the importance of quaternary structure of adiponectin *in vivo* (26), this study is the first to describe a robust *in-vitro* assay and a specific molecular effect that can distinguish HMW-adiponectin from lower molecular forms.

Atherothrombotic vascular disease in general, and plaque necrosis (*i.e.*, advanced lesional macrophage death) in particular, are substantially increased in subjects with obesity and insulin resistance, including metabolic syndrome and type 2 diabetes (40,41). While a number of mechanisms to explain this phenomenon can be envisioned, at least one may be the drop in plasma adiponectin in these subjects, leading to an increase in advanced lesional macrophage death. Indeed, the presence of adiponectin in atherosclerotic lesions reflects plasma levels (42), and there is an inverse relationship between plasma adiponectin and coronary artery disease in humans (27). Previous adiponectin transgenic and gene-targeted mouse studies have shown an inverse relationship between plasma adiponectin and the area of early-mid-stage aortic atherosclerotic lesions in mice (25). However, advanced lesional macrophage death and plaque necrosis were not reported, and the site of production, form, and levels of adiponectin in the transgenic studies were either non-physiologic or not reported. The data herein provide the rationale for a future transgenic study that involves native forms of adiponectin, notably the HMW form, and uses advanced lesional macrophage death and necrosis as the key endpoint.

In theory, the findings herein may also be applicable to aspects of obesity and insulin resistance. Macrophages are recruited to adipose tissue in the setting of obesity, and the resulting inflammatory response may play a role in obesity-associated insulin resistance (43). It is not yet known whether such macrophages are subjected to ER stress and, if so, whether this would be functionally important. However, other studies have shown that ER stress in liver and muscle can promote insulin resistance (44). Moreover, we have shown that UPR activation in macrophages induces the secretion of inflammatory cytokines, including TNFα, which can promote insulin resistance *in vivo* (45). Thus, a drop in the local concentration in adiponectin in adipose tissue in the setting of obesity may enhance UPR and inflammation in adipose macrophages, thus contributing to insulin resistance.

4. Figure legends:

Figure 1. HMW-adiponectin, but not LMW-adiponectin, inhibits FC-induced apoptosis. a, Macrophages were left untreated (control) or treated with 10 µg/ml HMW-adiponectin (HMW-ApN) for 24 h. The cells were then incubated for 24 h in the absence or presence of 50 µg/ml acetyl-LDL + 10 µg/ml 58035 to achieve FC loading (FC). The cells that were previously treated with adiponectin also received it during this 24-h FC loading period as well. The macrophages were stained with Alexa 488 Annexin V (green) and propidium iodide (red). Representative fluorescent images and quantitative apoptosis data from four fields of cells for each condition are shown. The data are expressed as the percent of total cells that stained with Annexin V and propidium iodide. Data are expressed as mean ± SEM (n=4). **b**, The same experimental design was used except LMW-adiponectin was used. Bar, 20 µm.

Figure 2. HMW-adiponectin, but not LMW-adiponectin, blocks CHOP induction by FC loading and by other inducers of the UPR. a, Macrophages were left untreated (control) or treated with 5 or 10 µg/ml HMW-adiponectin (HMW-ApN) for 24 h. The cells were then subjected to control or FC-loading conditions exactly as in Fig. 1. Whole cell

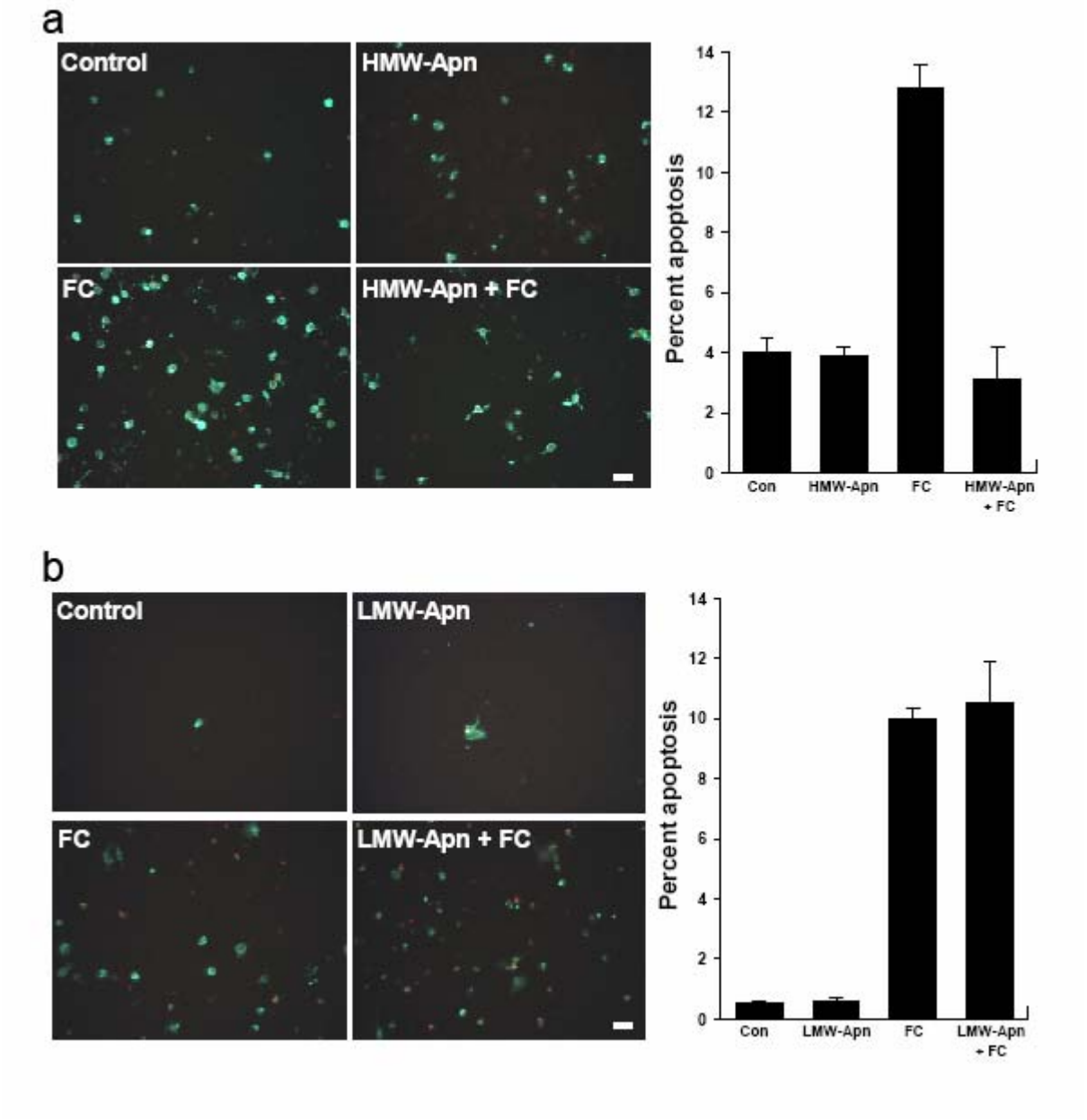
lysates were immunoblotted for CHOP and α-actin as a loading control. **b**, As in **a**, except 10 µg/ml LMW-adiponectin was used. **c**, Macrophages were left untreated (control) or treated for 24 h with the indicated concentrations of trimer-adiponectin, LMW-adiponectin, and HMW-adiponectin. The cells were then incubated for 5 h in the absence or presence of 5 µg/ml tunicamycin. Whole cell lysates were immunoblotted for CHOP. **d**, As in **c**, but only HMW-adiponectin was used, and some of the samples were incubated for 5 h with 1 µM thapsigargin instead of tunicamycin.

Figure 3. HMW-adiponectin suppresses distal, but not proximal, UPR molecules. **a**, **b**, Macrophages were left untreated (control) or treated with 10 µg/ml HMW-adiponectin (HMW-ApN) for 24 h. The cells were then incubated for 5 h in the absence or presence of 5 µg/ml tunicamycin. Nuclei-free cell extracts were immunoprecipitated and then immunoblotted with anti-IRE1 (**a**) or anti-PERK (**b**) antibody. Activated forms of IRE1 and PERK are phosphorylated (P-), resulting in an upward shift the band. **c**, Macrophages were incubated as in **a**, and the blots were probed using the indicated antibodies. The following cell preparations were used: whole cell lysates for phospho-eIF2α (α-actin control), and nuclear extracts for ATF4, XBP1, and CHOP (nucleophasmin control). For GADD34, nuclei-free cell extracts were immunoprecipitated and then immunoblotted with anti-GADD34 antibody. **d**, As in **c**, but some of the macrophages were loaded with FC (see Fig. 1) instead of tunicamycin to induce the UPR. Whole cells lysates were immunoblotted for ATF3.

Figure 4. HMW-adiponectin suppresses ATF4 translation. **a**, Macrophages were left untreated (control) or treated with 10 µg/ml HMW-adiponectin (HMW-ApN) for 24 h. The cells were then incubated for the indicated times in the absence or presence of 5 µg/ml tunicamycin. Total RNA was extracted and subjected to quantitative RT-PCR using primers for ATF4 and a control mRNA, 36B4. The data are expressed as the level of ATF4 mRNA relative to that of 36B4 (means ± SEM, n=4).

Figure 5. Endogenous, circulating adiponectin can suppress the UPR in macrophages *in vivo*. Wild-type and adiponectin-null mice were injected i.p. with 5 µg tunicamycin or vehicle control, and 5 h later, the macrophages were harvested from the peritoneum and assayed immediately for expression of phospho-eIF2α and CHOP by immunoblotting.

5. Figures (see following pages):


a
Control
HMW-Apn
FC
HMW-Apn + FC
Percent apoptosis
0
2
4
6
8
10
12
14
Con
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FC
HMW-Apn + FC
b
Control
LMW-Apn
FC
LMW-Apn + FC
Percent apoptosis
0
2
4
6
8
10
12
14
Con
LMW-Apn
FC
LMW-Apn + FC

Figure 1

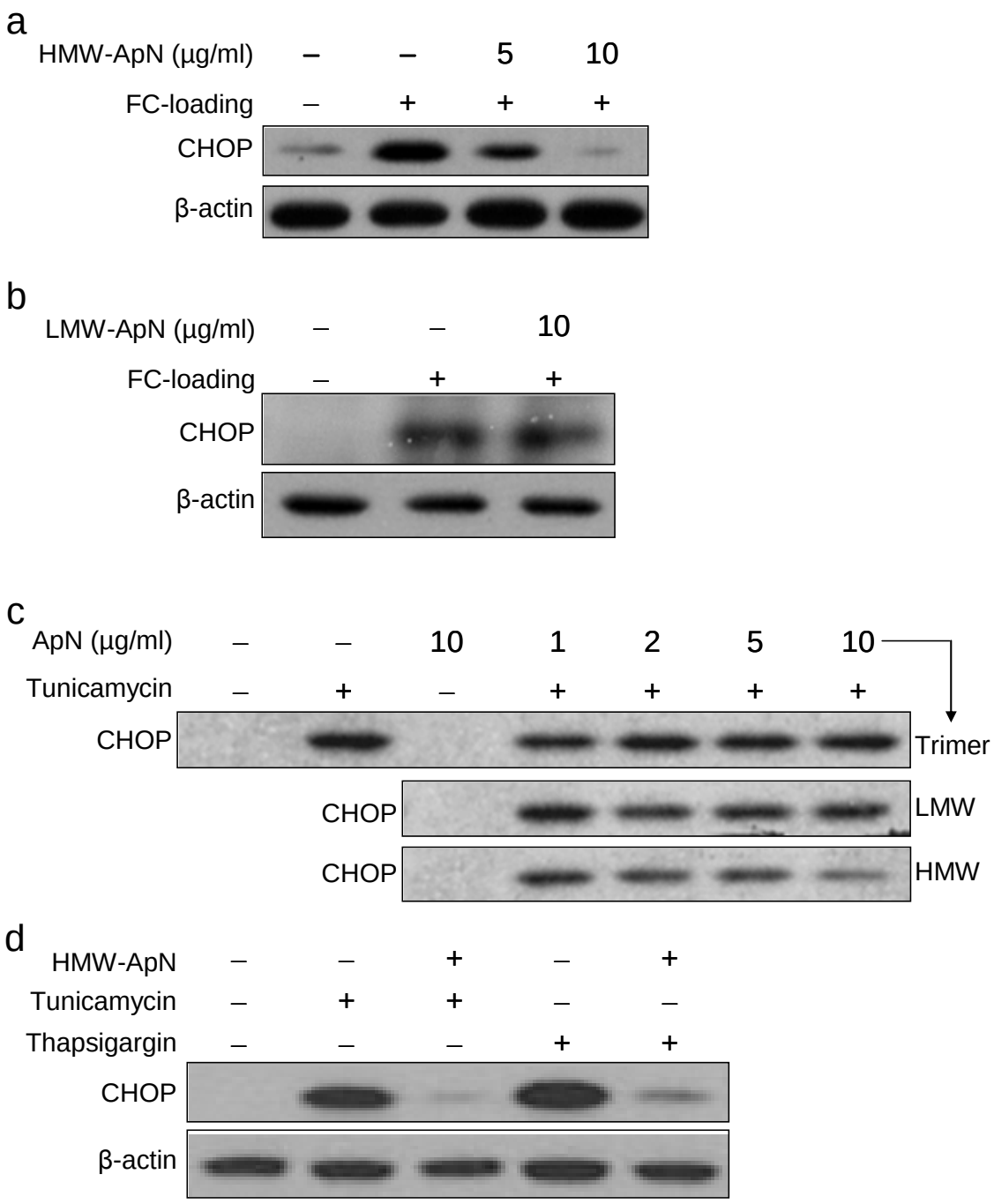
a
HMW-ApN (μg/ml) – – 5 10
FC-loading – + + +
CHOP
β-actin
b
LMW-ApN (μg/ml) – – 10
FC-loading – + +
CHOP
β-actin
c
ApN (μg/ml) – – 10 1 2 5 10
Tunicamycin – + – + + + +
CHOP
Trimer
CHOP
LMW
CHOP
HMW
d
HMW-ApN – – + – +
Tunicamycin – + + – –
Thapsigargin – – – + +
CHOP
β-actin

Figure 2

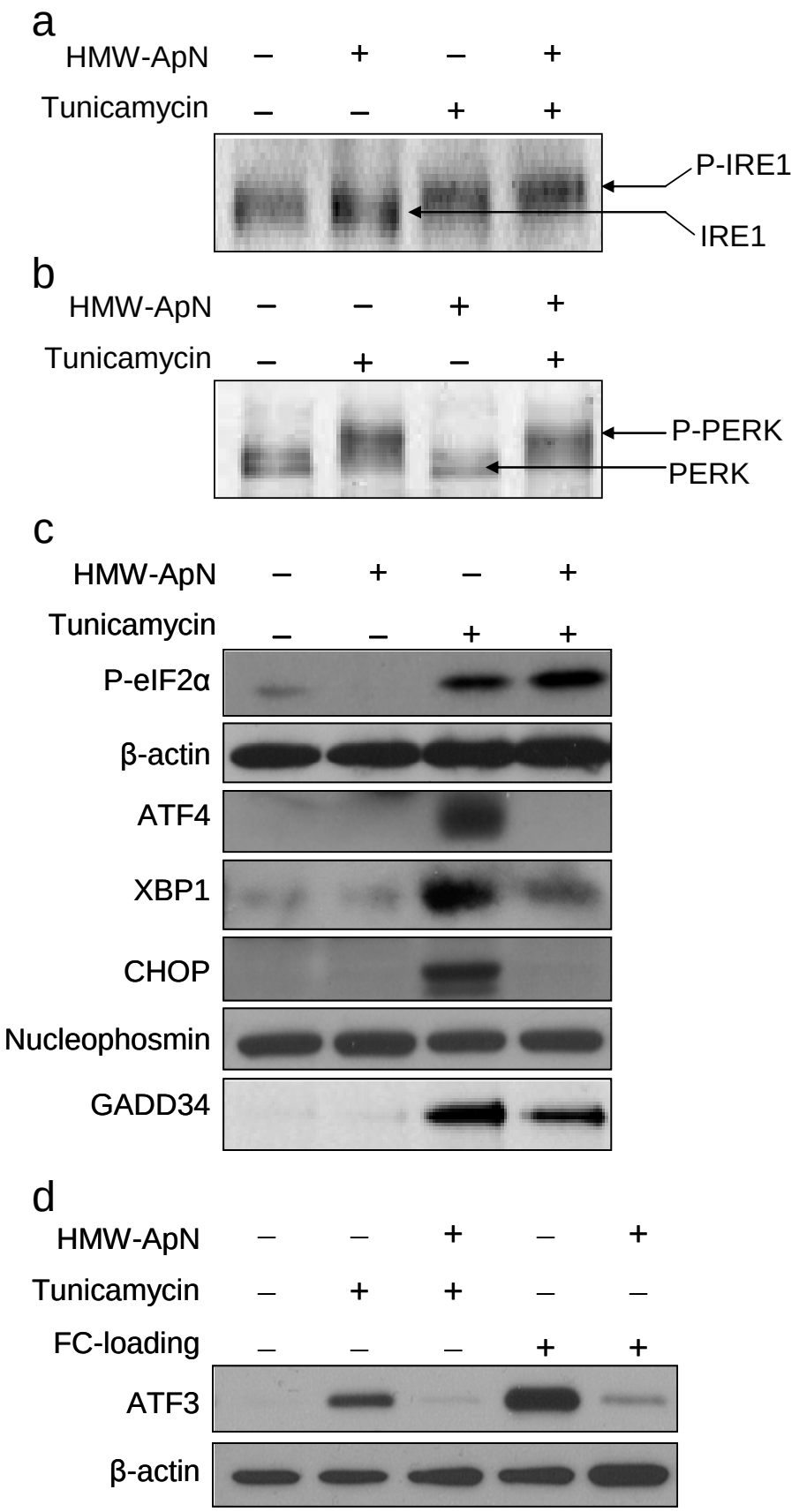
a
HMW-ApN – + – +
Tunicamycin – – + +
P-IRE1
IRE1
b
HMW-ApN – – + +
Tunicamycin – + – +
P-PERK
PERK
c
HMW-ApN – + – +
Tunicamycin – – + +
P-eIF2α
β-actin
ATF4
XBP1
CHOP
Nucleophosmin
GADD34
d
HMW-ApN – – + – +
Tunicamycin – + + – –
FC-loading – – – + +
ATF3
β-actin

Figure 3

a

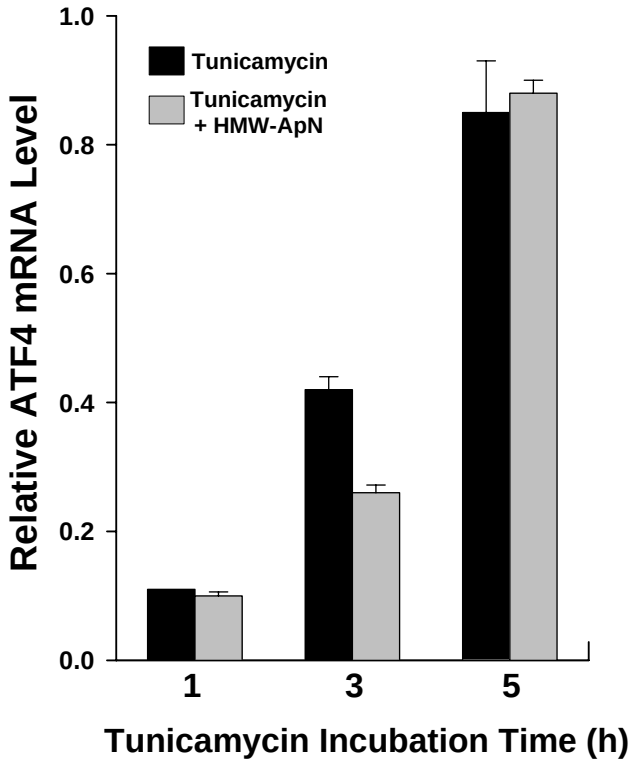
1.0
0.8
0.6
0.4
0.2
0.0
Relative ATF4 mRNA Level
Tunicamycin
Tunicamycin
+ HMW-ApN
1
3
5
Tunicamycin Incubation Time (h)

Figure 4

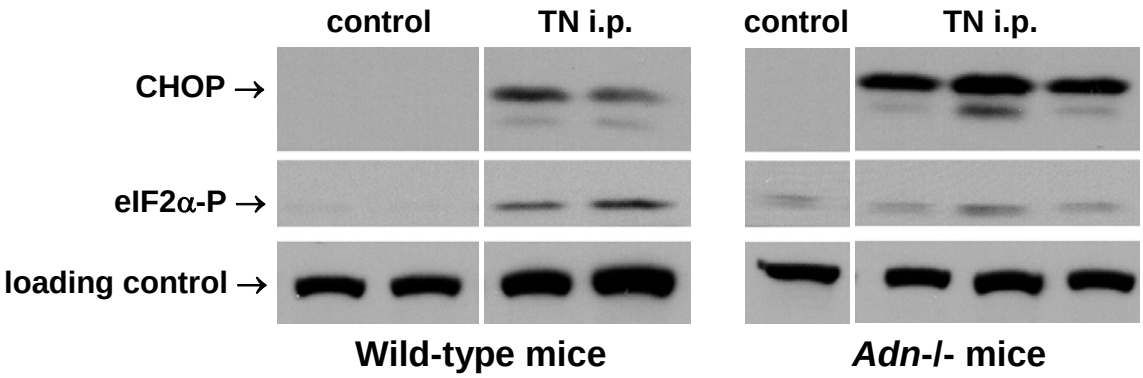
control
TN i.p.
control
TN i.p.
CHOP →
eIF2α-P →
loading control →
Wild-type mice
Adn-/- mice

Figure 5

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstration that TZDs, which are commonly used drugs for insulin resistance in obese individuals, promote macrophage apoptosis under conditions that are likely to be important in advanced atherosclerosis

- Demonstration that a widely used TZD, pioglitazone, promotes macrophage death and plaque necrosis in advanced atherosclerotic lesion *in vivo*

- Demonstration that the detrimental effects of TZDs on promoting macrophage death are PPARγ-independent, thus opening the opportunity to create a more beneficial TZD-like drug for obese. insulin-resistant individuals, *i.e.*, one that does not have this PPARγ-independent effect but that would still have the beneficial insulin-sensitizing PPARγ-dependent effects

- Demonstration that HMW-adiponectin protects macrophages from death under conditions that mimic those in advanced atherosclerotic lesions, those raising the possibility of an anti-atherosclerosis drug for obese individuals that mimics the action of adiponectin

- Demonstration that HMW-adiponectin suppresses a pro-apoptotic branch of the UPR by a mechanism that is unique, thus providing a molecular mechanism for the survival effect of the hormone on macrophages and also providing one of the first cellular-molecular *in-vitro* assays that is distinct for the HMW form of adiponectin

REPORTABLE OUTCOMES:

- Two manuscripts in preparation (these are incorporated in the progress report above)

- Abstract (poster) presented at the 2006 American Heart Association Scientific Sessions, Nov. 2006, Chicago, IL. **This abstract won the prize for best basic science poster on the day that it was presented.**

- Oral presentations on the material described in this progress report

- Training of a post-doctoral fellow, Dr. Dongying Cui, in the Tabas laboratory

CONCLUSIONS:

The studies completed as part of this project have revealed new cellular and molecular links between obesity/insulin resistance and atherothrombotic vascular disease. We have shown that a side-effect of a class of insulin-sensitizing PPARγ-activating drugs called TZDs, which are used widely in obese people, may promote vascular disease by

enhancing advanced atherosclerotic lesional macrophage death and plaque necrosis. We have also shown how adiponectin, a hormone that is decreased in obese subjects, might protect against vascular disease, namely, by suppressing advanced lesional macrophage death. Future plans will be directed at testing the relevance of these new concepts in advanced atherosclerosis in mouse models *in vivo*; relating these findings to our original observations regarding the pro-apoptotic role of angiotensin 2; and showing evidence of the fundamental macrophage pro-apoptotic signaling pathway—the UPR—in human vulnerable plaques. Our new advances this year suggest new ideas for drug therapy: a "super" TZD that would eliminate the potential pro-atherogenic effects of these drugs; and a mimetic for HMW-adiponectin, that would protect macrophages from advanced lesional apoptosis. If successful, these drugs would help stem the rising tide of obesity-related heart disease in middle-aged and older military personnel and their families as well as in the general population.

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12. Tabas,I. 2005. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. *Arterioscler.Thromb.Vasc.Biol* 25:2255-2264.

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21. Chawla,A., Barak,Y., Nagy,L., Liao,D., Tontonoz,P., and Evans,R.M. 2001. PPAR-γ dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. *Nat.Med.* 7:48-52.

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23. Fonseca,V., Jawa,A., and Asnani,S. 2006. Commentary: the PROactive study--the glass is half full. *J.Clin.Endocrinol.Metab* 91:25-27.

24. Tabas,I. 2004. Apoptosis and plaque destabilization: the role of macrophage apoptosis induced by cholesterol. *Cell Death & Differentiation* 11:S12-S16.

25. Kadowaki,T. and Yamauchi,T. 2005. Adiponectin and adiponectin receptors. *Endocr.Rev.* 26:439-451.

26. Pajvani,U.B., Du,X., Combs,T.P., Berg,A.H., Rajala,M.W., Schulthess,T., Engel,J., Brownlee,M., and Scherer,P.E. 2003. Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity. *J.Biol.Chem.* 278:9073-9085.

27. Pischon,T., Girman,C.J., Hotamisligil,G.S., Rifai,N., Hu,F.B., and Rimm,E.B. 2004. Plasma adiponectin levels and risk of myocardial infarction in men. *JAMA* 291:1730-1737.

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33. Harding,H.P., Zhang,Y., and Ron,D. 1999. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* 397:271-274.

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36. Jiang,H.Y. and Wek,R.C. 2005. Phosphorylation of the alpha-subunit of the eukaryotic initiation factor-2 (eIF2alpha) reduces protein synthesis and enhances apoptosis in response to proteasome inhibition. *J.Biol.Chem.* 280:14189-14202.

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38. Lu,P.D., Harding,H.P., and Ron,D. 2004. Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. *J Cell Biol* 167:27-33.

39. Lassot,I., Estrabaud,E., Emiliani,S., Benkirane,M., Benarous,R., and Margottin-Goguet,F. 2005. p300 modulates ATF4 stability and transcriptional activity independently of its acetyltransferase domain. *J.Biol.Chem.* 280:41537-41545.

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43. Bouloumie,A., Curat,C.A., Sengenes,C., Lolmede,K., Miranville,A., and Busse,R. 2005. Role of macrophage tissue infiltration in metabolic diseases. *Curr.Opin.Clin.Nutr.Metab Care* 8:347-354.

44. Ozcan,U., Cao,Q., Yilmaz,E., Lee,A.H., Iwakoshi,N.N., Ozdelen,E., Tuncman,G., Gorgun,C., Glimcher,L.H., and Hotamisligil,G.S. 2004. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 306:457-461.

45. Uysal,K.T., Wiesbrock,S.M., Marino,M.W., and Hotamisligil,G.S. 1997. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* 389:610-614.

APPENDICES:

(NB: draft manuscripts in preparation have been incorporated into the body of the progress report)

I. ABSTRACT (presented at the 2006 American Heart Association Scientific Sessions, Nov. 2006, Chicago, IL):

Published in Circulation Vol. 114, page II-25

Adiponectin, an Atherosclerosis-Protective Hormone, Inhibits Cholesterol-Induced Activation of the Unfolded Protein Response and Apoptosis in Macrophages

Jerry Arellano, Philipp Scherer, David Ron, Benoit Viollet, Larry Shapiro, Troy Burke, Ira Tabas; Columbia University Medical Center, Albert Einstein Medical Center, & NYU Medical Center, New York, NY; Institut Cochin, Paris, France

In advanced atherosclerosis, macrophage (Mϕ) apoptosis leads to plaque necrosis and destabilization. Mϕ apoptosis in this setting can be triggered by excess free cholesterol (FC), which overloads the endoplasmic reticulum (ER) with FC and triggers a stress pathway known as the Unfolded Protein Response (UPR). Prolonged FC-induced UPR activation triggers apoptosis via the effector CHOP. Adiponectin (ApN) is an abundant adipocyte-derived hormone whose plasma levels fall in obesity/insulin resistance. Most importantly, ApN is found in atherosclerotic lesions, and low levels are associated with coronary artery disease in humans and atherosclerosis in mice. In studies exploring possible athero-protective mechanisms of ApN, we found that ApN is a potent suppressor of FC-induced Mϕ apoptosis. Remarkably, this action was specific for the naturally occuring high-molecular-weight form of ApN, which is the form that promotes insulin sensitization *in vivo*. The mechanism was not due to suppression of FC uptake or FC trafficking to the ER. Rather, ApN was a potent suppressor of FC-induced CHOP as well as CHOP induced by other UPR activators. Whereas most CHOP-suppressing agents function by relieving global ER stress at the level of the upstream UPR activators PERK and IRE1, ApN suppresses CHOP by uniquely down-regulating the intermediate UPR mediator ATF4. ApN does not suppress ATF4 mRNA or accelerate ATF4 degradation, indicating translational control. While ATF4-CHOP suppression likely explains at least part of the anti-apoptotic action of ApN, we found that ApN also potently activates STAT3, a known survival effector. Thus, ApN inhibits FC-induced Mϕ apoptosis, most likely by a combination of ATF4-CHOP suppression and STAT3 activation. These findings shed new light on how ApN affects Mϕ biology, with potential relevance to the athero-protective effects of this adipocyte hormone.

II. Curriculum vitae:

Personal data

Name: Ira Abram Tabas
Birth date: April 22, 1953
Birthplace: Philadelphia, Pennsylvania
Citizenship: USA
Office address: Department of Medicine, PH 8East-105F, Columbia University
630 West 168th Street, New York, NY 10032
Contact information: Tel: 212-305-9430; Fax: 212-305-4834; E-mail: iat1@columbia.edu

Academic training

Undergraduate:	Tufts University (Medford, MA), 1975, B.S.
Graduate:	Washington University (St. Louis, MO), 1981, M.D., Ph.D. (Biochemistry)
Ph.D. Thesis:	"The Processing of Asparagine-Linked Oligosaccharides During Glycoprotein Biosynthesis"; Dr. Stuart Kornfeld, Sponsor
M.D. Licensure:	State of New York (#150522)

Traineeship

Internship/Residency:	Internal Medicine, Columbia-Presbyterian Medical Center, New York, NY (1981-1983)
Clinical Fellowship:	Endocrinology/Metabolism, Columbia-Presbyterian Medical Center, New York, NY (1983-1985)
Research Fellowship:	Laboratory of Dr. Alan Tall, Department of Medicine, Columbia University, New York, NY, 1983-1985

Board certification

Internal Medicine, 1985
Endocrinology/Metabolism, 1987

Professional organizations and societies

Arteriosclerosis Council (Arteriosclerosis, Thrombosis, and Vascular Biology as Council as of 1997) of the American Heart Association; appointed member of the Membership/ Credentials Committee (1990-1992, 1997-1999) and Program Committee (1992-1994; 2000-2002)
American Society of Biochemists and Molecular Biologists
American Association for the Advancement of Science
American Society for Cell Biology
New York Lipid Club
American Society for Clinical Investigation
Interurban Clinical Club
American Association of Physicians

Academic appointments

Assistant Professor of Medicine, Columbia University College of Physicians and Surgeons, New York, NY (1985-1992)
Assistant Professor of Anatomy & Cell Biology, Columbia University College of Physicians and Surgeons, New York, NY (1988-1992)
Associate Professor of Medicine and Anatomy & Cell Biology (**Tenured)**, Columbia University College of Physicians and Surgeons, New York, NY (1992-1997)
Professor of Medicine and Anatomy & Cell Biology (**Tenured)**, Columbia University College of Physicians and Surgeons, New York, NY (1997-present)
Professor of Physiology and Cellular Biophysics (**Tenured**), Columbia University College of Physicians and Surgeons, New York, NY (2004-present)
Vice-Chairman of Research, Department of Medicine, Columbia University (2004-present)

Hospital appointments

Assistant Attending Physician of Medicine, Columbia-Presbyterian Medical Center, New York, NY (1985-1992)
Associate Attending Physician of Medicine, Columbia-Presbyterian Medical Center, New York, NY (1992-present)

Attending Physician of Medicine, Columbia-Presbyterian Medical Center, New York, NY (1997-present)

Honors

Phi Beta Kappa, Tufts University, Medford, MA (1974)

Summa cum laude, Tufts University, Medford, MA (1975)

Letter of Commendation, Washington School of medicine, St. Louis, MO (1977)

Mosby Scholarship Book Award, Washington University School of Medicine (1981)

Alpha Omega Alpha, Washington University School of Medicine, St. Louis (1981)

Pfizer Research Award for Young Faculty (1985-1987)]

Silberberg Assistant Professorship of Medicine, Columbia University (1988-1993)

American Heart Association Established Investigator Award (1988-1993)

Invited speaker to Gordon Research Conferences (1988-1989, 1994, 1996, 1997, 1999-2002, 2004, 2006), Kern Lipid Conference (1989, 1992, 1998, 2000, 2004); Deuel Research Conference (1997, 2002, 2004); International Symposium on Atherosclerosis (1997, 2000, 2003); National AHA and ATVB meetings (2000-2005); session chair in satellite symposium of International Congress of Biochemistry and Molecular Biology (1997), Satellite symposium of the American Society for Biochemistry and Molecular Biology (1998); Keystone Conferences on Cellular and Molecular Events in the Pathogenesis of Atherosclerosis (2001); Regulatory and Effector Functions of Macrophages (2003); and Cellular Biology of Atherosclerosis (2004)

Doctor Harold and Golden Lamport Research Award (1990)

Elected to the American Society for Clinical Investigation (1992)

Scientific Board of the Stanley J. Sarnoff Endowment for Cardiovascular Science, Inc. (1992-1996)

Editorial Board of *Journal of Biological Chemistry* (1995-2000)

Elected to Interurban Clinical Club (1996-present)

Elected to American Association of Physicians (1998-present)

Deputy Editor, *Journal of Clinical Investigation*, (2002-2007)

American Heart Association/ATVB Council Special Recognition Award (2003)

Named Chair, Department of Medicine (Richard J. Stock Professor of Medicine)

Chairman, 2005 Keystone Symposium on the Cellular Biology of Atherosclerosis

External Advisory Committee, Deuel Research Conferences (2004-2009)

Scientific Board, Kern Lipid Conference (2005-2010)

David Rubinstein Lectureship of the Canadian Lipoprotein Conference (2005)

Closure Lectureship of the 10th Scientific Symposium of the Lilly Foundation entitled "Nutrition, Lipids and Atherosclerosis", El Escorial, Madrid (2006)

Chairman, 2010 Gordon Conference on Lipoprotein Metabolism

Fellowship and grant support

Past:

Fellowship: NIH training grant (NHLBI)**,** 1983-85, trainee

Pfizer Research Award for Young Faculty, 1985-1987, Principal Investigator, $50,000 per annum

Project of NIH SCOR Grant in Atherosclerosis (NHLBI), 1986-1990, Co-Investigator, $104,000 per annum

Project of NIH SCOR Grant in Atherosclerosis (NHLBI), 1991, Responsible Investigator, $90,000 per annum

Biomedical Research Support Grant (NIH), 1990-1991, Principal Investigator, $5,000

American Heart Association Established Investigatorship Award, 1988-1993, Principal Investigator, $35,000 per annum

New York Heart Association Grant-in-Aid, Principal Investigator, 1992-1995, $42,000 per annum

Research Supplement for Minority Individuals in Postdoctoral Training (Dr. Anselm K. Okwu)

American Heart Association, New York City Affiliate, Participating Laboratory Award (Dr. Yoshimune Shiratori)

Postdoctoral Fellowship Award in Atherosclerosis (Dr. Paul Skiba)

Schering-Plough Research Grant, 1989-1995, Responsible Investigator, $50,000 per annum

Individual National Research Supplement Award for Postdoctoral Training (Dr. G. Andrew Keesler)

Postdoctoral Fellowship Award in Nutrition (Dr. Sudhir Marathe)

NIH R01 grant (NHLBI), Principal Investigator, 1992-1997, $120,000 per annum

Project of NIH SCOR Grant in Atherosclerosis (NHLBI), Responsible Investigator, 1991-1996, $105,000 per annum

Council for Tobacco Research Award, Principal Investigator, 1995-1998, $75,000 per annum

Postdoctoral Fellowship Award in Atherosclerosis (Dr. Wei Tang)

NIH R01 grant (NHLBI), Principal Investigator, 2001-2004, $215,000 per annum

Research grant from Berlex Laboratories, 2003-2004, $100,000 per annum

AHA Heritage Affiliate Postdoctoral Training Grant, 2004-2005 (Dr. Tracie DeVries)

Present:

NIH P01 grant (NHLBI), Responsible Investigator, 2006-2011, $297,000 per annum
NIH SCOR Grant in Vascular Biology (NHLBI), Responsible Investigator or Project and Pathology Core, 2002-2007, $282,000 per annum
NIH R01 grant (NHLBI), Principal Investigator, 2003-2007, $250,000 per annum
NIH R01 grant (NHLBI), Principal Investigator, 2005-2009, $250,000 per annum
Department of Defense grant (USAMRMC), Principle Investigator, 2006-2010, $250,000 per annum
NIH Postdoctoral Fellowship Award (Dr. Tracie DeVries-Seimon)
NIH Postdoctoral Fellowship Award in Atherosclerosis (Dr. Edward Thorp)
AHA Heritage Affiliate Postdoctoral Training Grant, 2005-2007 (Dr. Wahseng Lim)
Merck Sponsored Research Project, Principal Investigator, 2004-2006, $100,000 per annum

Departmental and university committees

Faculty advisor for Columbia University College of Physicians and Surgeons medical students (1986-1994)
Member of the Columbia University Research Advisory Committee for first year medical student summer research projects (1990)
Member of the Department of Medicine Resident Selection Committee (1990-present)
Organizer of the Department of Medicine Young Faculty Research Conference (1990-1992)
Member of Department of Medicine Subcommittee on Research (1991) and Committee for Organizing Departmental Retreat (1995)
Member of Doctoral Program Subcommittee on Nutrition (1991-present)
Co-Director of Basic Research Track of the CPMC Internal Medicine Residency Program (1992-1997),
Scientific Advisory and Executive Committee, Medical Scientist Training (MD-PhD) Program, Columbia University (1993-present)
Member, Curriculum Committee of the College of Physicians & Surgeons (1997-2002)
Co-Associate Director, Medical Scientist Training (MD-PhD) Program, Columbia University (2001-present)
Chairman, Committee on Promotions of the Department of Medicine (1997-2004)
Member of Search Committees for Director of Pathology, St. Luke's Roosevelt Hosp. (1992), Chairperson of the Department of Pharmacology, Columbia University (1994-1995), Chairperson-Division of Cardiology, Columbia University (1999), Director of the Irving Center for Cancer Research (2004), Chairperson-Division of Oncology (2005)

Teaching experience and responsibilities

Specific courses:

Medical Student Preceptor (1989, 1991, 1994, 1996), 6 students
Abnormal Human Biology, Atherosclerosis session preceptor (1987-present), 30 students
Cellular Membranes graduate course (Department of Anatomy & Cell Biology), LDL receptor and intracellular cholesterol metabolism sessions (1987-present), 30 students
Pharmacology graduate student course, LDL receptor session (1989-1993), 20 students
Histology medical student course, microcirculation session (1989-1994), 200 students
Advanced pathophysiology course for fourth year medical, atherosclerosis sessions (1990- 1996), 40 students
Pathology graduate student course (Molecular Mechanisms of Disease), organizer and lecturer of Atherosclerosis section (1991-present), 15 students
Science Basic to the Practice of Medicine (formerly Biochemistry of Disease) medical student course, Atherosclerosis session (1992-present), 120 students
Pathophysiology course for 2nd-year medical students, Atherosclerosis session (1997-present), 120 students
Molecular and Cellular Biology of Nutrients, Apoptosis section (2001-), 15 students
Molecular and Cellular Cardiology Lecture Series, Transgenic Models section (1998-), 15 fellows

General teaching activities:

Attending on Internal Medicine ward service (1985-present), 2-3 students and 3 housestaff physicians
Attending on Endocrinology ward service (1987-present), 1-2 students and 1 fellow
Attending in Combined Endocrine/Diabetes, Thyroid, and Lipid Clinics (1987-present), 1-2 students and 1-2 clinical fellows

Ph.D. Thesis sponsor:

Lori Bottalico, Department of Anatomy/Cell Biology, Columbia University (1989-1992)
Scott Schissel, Department of Anatomy/Cell Biology, Columbia University (1993-1997)
Andrew Leventhal, Department of Anatomy/Cell Biology, Columbia University (2000-2004)—Winner of the 2004 Samuel W. Rover and Lewis Rover Award for Scholarship and Outstanding Achievement in Anatomy and Cell Biology

Masters thesis sponsor:

Sungtae Lim, Institute of Human Nutrition, Columbia University (1989)
Woan-Chyng Su, Institute of Human Nutrition, Columbia University (1990)

Ph.D. Advisory/Examination committees:

Deborah A. Lazzarino, Department of Anatomy/Cell Biology, Columbia University (Ph.D. advisory committee and examination, 1987-1990)
Shing-Jong Lin, Department of Physiology, Columbia University (Ph.D. examination, 1989)
Maria Davila-Bloom, Institute of Human Nutrition, Columbia University (Ph.D. examination, 1989)
Fan Yuan, Department of Engineering, The City University of New York (Ph.D. examination, 1990-1993)
Lester S. Johnson, Department of Pathology, Columbia University, Ph.D. thesis committee (1990-1993)
Steven Rumsey, Institute of Human Nutrition, Ph.D. thesis committee (1992-1993)
Thomas E. Phalen, Albert Einstein College of Medicine, Ph.D. thesis defense committee (1993)
Sripriya Chari, Integrated Program in Cellular, Molecular, and Biophysical Studies, Qualifying Examination (1993)
Zhenglun Zhu, Department of Anatomy/Cell Biology, Columbia University (Ph.D. advisory committee and examination, 1991-1993)
Lori Masucci, Institute of Human Nutrition, Ph.D. thesis committee (1993-1996)
Cory Huang, Department of Pathology, Ph.D. thesis committee (1995)
Mingyue Zhou, Institute of Human Nutrition, Ph.D. thesis committee (1995-)
Hong-yuan Yang, Institute of Human Nutrition, Ph.D. thesis committee (1995-)
Donata Paresce, Department of Pathology, Ph.D. thesis committee (1997)
Furcy Paultre, Institute of Human Nutrition, Ph.D. thesis committee (1997-)
Chris William, Integrated Program. Ph.D. qualifying exam (1997)
Nrgo Storey, Department of Biochemistry, Dalhousie University, Ph.D. examination, 1997
Peter Sartipy, Wallenberg Laboratory, University of Gothenburg, Sweden, opponent, 2000
Ying Lui, Institute of Human Nutrition, Ph.D. thesis committee (1999-)
Edward Cha, Department of Microbiology, Ph.D. thesis committee (2000-)
Yu Sun, Institute of Human Nutrition, Ph.D. thesis committee (1997-2002)

Other professional activities

Reviewer of over 3000 manuscripts for *Journal of Clinical Investigation, Journal of Biological Chemistry, Journal of Lipid Research, Arteriosclerosis, and Biochimica Biophysica Acta* (1985-present)
Ad hoc grant reviewer for National Science Foundation (1989-present)
Grader for research abstracts submitted to the American Heart Association Annual Meeting (1990, 1992-1994, 1998)

Sub-group reviewer for American Heart Association Established Investigator and Clinical Scientist Award grants (1991 & 1992)
Member of American Heart Association grant-in-aid study section (1992-1993)
Member Scientific Board of the Stanley J. Sarnoff Endowment for Cardiovascular Science, Inc. (1992-1996)
Vice-chairman of American Heart Association grant-in-aid study section (1994)
Consultant for Merck, Schering-Plough, Warner-Lambert, Berlex, Eli Lilly, Pfizer, Talaria Biotech, ReddyUS, Amersham/GE, and Bristol-Myers-Squibb, Novartis, Sankyo
Institutional representative for the American Society of Clinical Investigation (1998-2000)
Co-Editor of October 2000 and 2001 issues of Current Opinion in Lipidology
Organizer and Chairman, Keystone Conference on the Cellular Biology of Atherosclerosis (2005)
External Advisory Committee, Deuel Research Conferences (2004-2009)
Scientific Board, Kern Lipid Conference (2005-2010)
General Council and Review Panel for Future Leaders Grant Program, The Leadership Council for Improving Cardiovascular Care (2005-)

Publications (* indicates that Dr. Tabas is a senior author [*i.e.*, post-graduate school] *and* had a major role in the publication)

Original, peer-reviewed articles:

1. Tabas, I., Schlesinger, S. and Kornfeld, S. (1978) Processing of high mannose oligosaccharides to form complex type of oligosaccharides on the newly synthesized polypeptides of the vesicular stomatitis virus G protein and the IgG heavy chain. *J. Biol. Chem.* **253**:716-722.

2. Li, E., Tabas, I. and Kornfeld, S. (1978) The synthesis of complex type of oligosaccharides. I. Structure of the lipid-linking oligosaccharide precursor of the complex type oligosaccharides of the vesicular stomatitis virus G. protein. *J. Biol. Chem*. **253**:7762-7770.

3. Kornfeld, S., Li, E. and Tabas, I. (1978) The synthesis of complex type oligosaccharides. II. Characterization of the processing intermediates in the synthesis of the complex oligosaccharide units of the vesicular stomatitis virus G protein. *J. Biol. Chem*. **253**:7771-7778.

4. Tabas, I., and Kornfeld, S. (1978) The synthesis of complex type oligosaccharides. III. Identification of an α-D-mannosidase activity involved in a late stage of processing of complex type oligosaccharides. *J. Biol. Chem*. **253**:7779-7786.

5. Tabas, I. and Kornfeld, S. (1979) Purification and characterization of a rat liver Golgi α-mannosidase capable of processing asparagine-linked oligosaccharides. *J. Biol. Chem*. **254**:11655-11663.

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Reviews, chapters, and letters-to-editor:

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