[Frontiers in Bioscience 12, 2291-2298, January 1, 2007]

Plasma insulin levels predict the development of atherosclerosis when IRS2 deficiency is combined with severe hypercholesterolemia in apolipoprotein E-null mice

Herminia Gonzalez-Navarro [1], Marian Vila-Caballer [1], Maria F. Pastor [2], Angela Vinue [1], Morris F. White [3], Deborah Burks [2], Vicente Andres [1]

[1] Vascular Biology Laboratory, Department of Molecular and Cellular Pathology and Therapy, Instituto de Biomedicina de Valencia (IBV-CSIC), Valencia, Spain, [2] Centro de Investigacion Principe Felipe, Valencia, Spain, [3] Howard Hughes Medical Institute, Children's Hospital Boston, Harvard Medical School, Boston, Massachusetts, USA

TABLE OF CONTENTS

1. ABSTRACT

Atherosclerosis is increased in type 2 diabetic patients but the precise mechanisms underlying this predisposition remain vague. Mice deficient for insulin receptor substrate 2 (IRS2) develop type 2-like diabetes and thus, provide a model to explore the molecular connection between deranged carbohydrate metabolism and atherosclerosis. To explore the relationship between defective insulin signalling and atherosclerosis, we have examined the development of atherosclerosis in the following groups of fat-fed mice: wild-type, diabetic *Irs2*-null (*Irs2$^{-/-}$*), atherosclerosis-prone apolipoprotein E-null (*apoE$^{-/-}$*), and doubly-deficient *apoE$^{-/-}$Irs2$^{-/-}$*. Surprisingly, glucose levels of *apoE$^{-/-}$Irs2$^{-/-}$* mice were comparable to those seen in wild-type and *apoE$^{-/-}$* and significantly lower than in *Irs2$^{-/-}$* mice. *Irs2$^{-/-}$* and *apoE$^{-/-}$Irs2$^{-/-}$* were hyperinsulinemic compared to wild-type and *apoE$^{-/-}$* mice. Atherosclerotic lesions were barely detectable in wild-type and *Irs2$^{-/-}$* mice, which displayed moderate hypercholesterolemia (~280 mg/dL). Notably, atherosclerosis was significantly enhanced in *apoE$^{-/-}$Irs2$^{-/-}$* compared with *apoE$^{-/-}$* mice, although both models displayed similar levels of severe hypercholesterolemia (>600 mg/dL). Circulating insulin levels predicted atherosclerotic lesion burden in *apoE$^{-/-}$Irs2$^{-/-}$* mice. Our results suggest that hyperinsulinemia as a result of *Irs2* genetic ablation contributes to increased atherosclerosis when combined with severe hypercholesterolemia in the absence of hyperglycaemia (*apoE$^{-/-}$Irs2$^{-/-}$* mice), thus implicating IRS2 as an important modulator of murine hypercholesterolemia-dependent atherosclerosis. Future studies are necessary to determine whether IRS2 dysfunction may promote atherosclerosis in normoglycemic, pre-diabetic patients with clinical manifestations of hyperinsulinemia and insulin resistance.

2. INTRODUCTION

Diabetes mellitus affects approximately 100 million persons worldwide, with 90-95% of these patients suffering from type 2 diabetes (1). Diabetic patients have a 2 to 10-fold higher risk of atherosclerosis and cardiovascular disease than the general population (1,2). Type 2 diabetes and the metabolic syndrome are characterized by hyperglycemia, hyperinsulinemia, insulin resistance, obesity, hypertension and dyslipidemia. These factors produce vascular disorders which facilitate atherosclerotic lesion formation and plaque instability, and additionally, favor formation and persistence of thrombi (1,3-5). Both type 2 diabetes and metabolic syndrome are predicted to increase at alarming rates during the next 50 years due to the prevalence of unhealthy lifestyle habits (e. g., obesity and lack of physical exercise). Therefore, it is of utmost importance to develop relevant animal models to be used in unravelling the mechanisms which accelerate atherosclerosis in pre-diabetic individuals (eg., hyperinsulinemic, glucose intolerant) and diabetic patients.

Disruption of the *Irs2* gene in the mouse produces pathological alterations very similar to type 2 diabetes and metabolic syndrome, including insulin resistance, hyperinsulinemia, glucose intolerance, obesity, hypertension, and moderate hyperlipidemia (6-9). Analysis of *Irs2*$^{-/-}$ mice has revealed that IRS2 signals are required for proper development and function of pancreatic β-cells as well as for the central regulation of appetite (10,11). Indeed, beta cell-specific overexpression of IRS2 promotes beta cell growth, survival, and insulin secretion that prevents diabetes in *Irs2*$^{-/-}$, obese and streptozotocin-treated mice (12).

These studies suggest that the *Irs2*$^{-/-}$ model provides an appropriate experimental setting to explore the influence of deranged carbohydrate metabolism on atherosclerosis. Thus, we intercrossed *Irs2*$^{-/-}$ with the atherosclerosis-prone *apoE*$^{-/-}$ mouse which spontaneously develops hypercholesterolemia and complex atherosclerotic lesions resembling those observed in humans and which can be accelerated by a high-fat cholesterol-rich diet (13). In this study, we have examined diet-induced atherosclerosis in wild-type, *Irs2*$^{-/-}$, *apoE*$^{-/-}$, and doubly-deficient *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice.

3. MATERIALS AND METHODS

3.1. Mice, genotyping and diet

Care of animals was in accordance with institutional guidelines. Male *Irs2*$^{-/-}$(C57BL/6J) (6) and female *apoE*$^{-/-}$ (C57BL/6J, Charles River) mice were crossbed to generate *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice. Genotyping for *Irs2* and *apoE* was done by PCR analysis as previously described (6) and as indicated by The Jackson Laboratory (http://jaxmice.org), respectively. After weaning, mice were maintained on a low-fat standard diet (2.8% fat; Panlab, Barcelona, Spain). At 2 months of age, mice received for 4 weeks an atherogenic diet containing 10.8% total fat, 0.75% cholesterol (S4892-E010, Ssniff, Soest, Germany).

3.2. Metabolic measurements

All measurements were performed with plasma of mice that were fasted overnight. Cholesterol, triglyceride and glucose levels were measured using enzymatic procedures (WAKO, St. Louis, USA). HDL-C and non-HDL-C were quantified after precipitation with Dextran-sulphate $MgCl_2$ (SIGMA, St. Louis, USA) as described (14). Insulin levels were quantified by ELISA (Mercodia, Sweeden). For glucose tolerance test, mice were injected with glucose (intraperitoneally, 2g/Kg of body weight) and plasma glucose levels were measured during 2 hours.

3.3. Atherosclerosis quantification

Fat-fed mice were killed, and after perfusion *in situ* with PBS followed by 4% paraformaldehyde/PBS, the heart and aorta were removed and fixation continued for 24h. A researcher blinded to genotype quantified the extent of atherosclerosis by computer-assisted morphometric analysis of whole-mounted aortic arch and thoracic aorta stained with Oil Red O (0.2% Oil Red O in 80% MeOH), and of aortic root cross-sections (intima-to-media ratio) as previously described (15). Differences in lesion area between males and females were not significant, therefore data from both sexes are shown.

3.4. Immunohistochemical staining

Vascular smooth muscle cells (VSMCs) were identified with monoclonal alkaline phosphatase-conjugated anti-smooth muscle alpha-actin (SM alpha-actin) antibody (clone 1A4, 1/20 dilution, a-5691, SIGMA) using Fast Red substrate (SIGMA). Macrophages were identified with a rat monoclonal anti-Mac 3 IgG1 antibody (clone M3/84, 1/200 dilution, sc-19991; Santa Cruz Biotechnology, California, USA), using a biotin-conjugated goat anti-rat IgG-B secondary antibody (sc-2041, Santa Cruz Biotechnology) and the ABC kit (VECTOR, Burlingame, USA) using DAB substrate (SEROTEC, Oxford, UK). Slides were counterstained with hematoxylin.

3.5. Statistics

Data are presented as mean±SE. Differences among groups were evaluated by 1-way ANOVA with Fisher's *post-hoc* test (Statview, SAS institute, Cary, USA). The *F*-test was used to measure the quality (significance) of regression analysis. Statistical significance was taken at $p \leq 0.05$.

4. RESULTS

4.1. Analysis of plasma glucose, insulin and lipid levels

To investigate the influence of deranged carbohydrate metabolism on diet-induced atherosclerosis, we examined the following groups of mice challenged for 4 weeks with an atherogenic diet enriched with fat and cholesterol: wild-type (WT), *Irs2*$^{-/-}$, *apoE*$^{-/-}$, and *apoE*$^{-/-}$ *Irs2*$^{-/-}$. Consistent with published observations in the C57BL/6 background (6), many *Irs2*$^{-/-}$ died from diabetic complications between 12 and 16 weeks of age; a similar proportion of *apoE*$^{-/-}$*Irs2*$^{-/-}$ animals also died during the same phase of our study. Glucose levels in fat-fed WT,

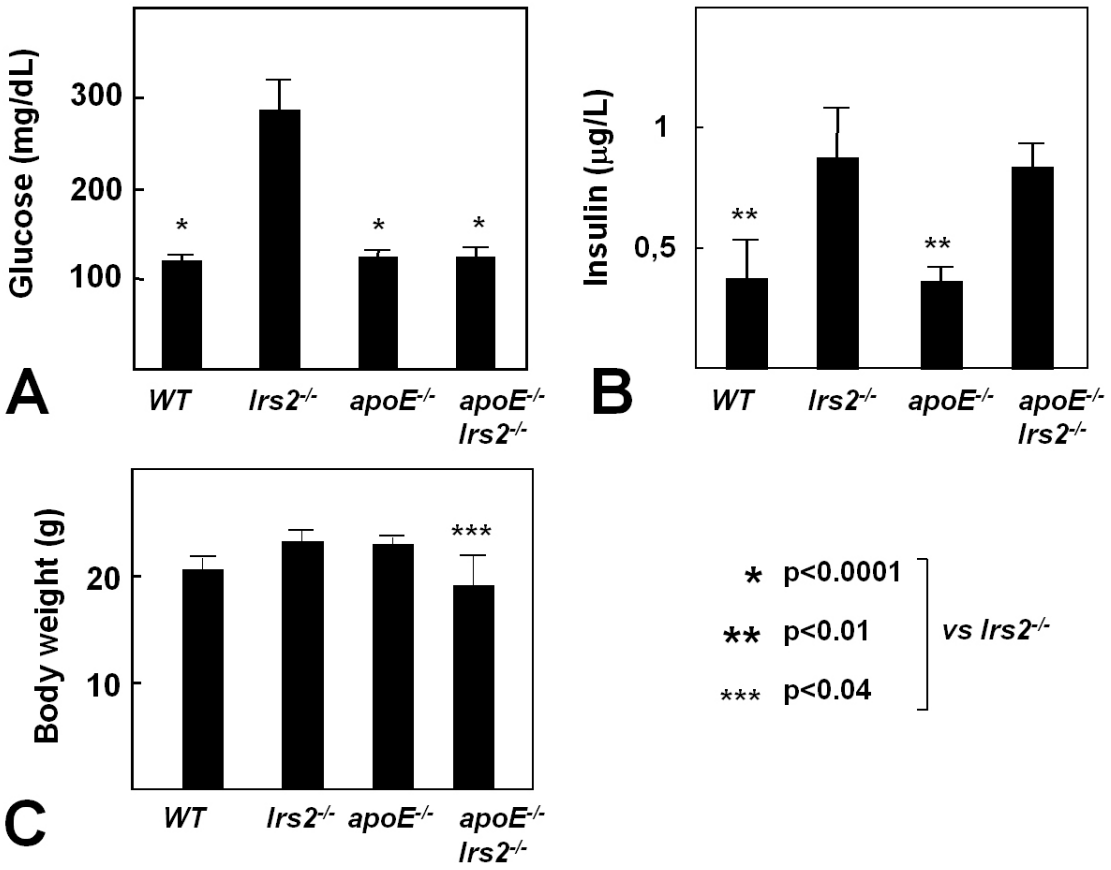

Figure 1. Analysis of body weight and plasma glucose and insulin levels. Mice received the atherogenic diet for 4 weeks. (A) Glucose level in WT (n=14, 6 females, 8 males), *Irs2*$^{-/-}$ (n=8, 4 females, 4 males), *apoE*$^{-/-}$ (n=18, 7 females, 11 males) and *apoE*$^{-/-}$*Irs2*$^{-/-}$ (n=7, 3 females, 4 males) mice. (B) Insulin level in WT (n=7, 2 females, 5 males) *Irs2*$^{-/-}$ (n=6, 3 females, 3 males), *apoE*$^{-/-}$ (n=10, 5 females, 5 males) and *apoE*$^{-/-}$*Irs2*$^{-/-}$ (n=6, 3 females, 3 males) mice. (C) Body weight in WT (n=14, 6 females, 8 males), *Irs2*$^{-/-}$ (n=8, 4 females, 4 males), *apoE*$^{-/-}$ (n=10, 4 females, 6 males) and *apoE*$^{-/-}$*Irs2*$^{-/-}$ (n=6, 3 females, 3 males) mice. Results represent the mean±SE.

apoE$^{-/-}$ and *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice were comparable but significantly lower than those in hyperglycaemic *Irs2*$^{-/-}$ mice (Figure 1A). Fasting insulin levels were indistinguishable between *Irs2*$^{-/-}$ and *apoE*$^{-/-}$*Irs2*$^{-/-}$, but significantly higher than in WT and *apoE*-/- mice (Figure 1B). Fat-fed animals from all groups exhibited glucose intolerance, which was more severe in *Irs2*$^{-/-}$ mice (data not shown). Body weight in *apoE*$^{-/-}$ and *Irs2*$^{-/-}$ mice was comparable and higher than that of *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice (Figure 1C).

As expected, plasma cholesterol level under standard chow was lower in both groups of mice with intact apoE (WT: 98.8±4.1 mg/dL, *Irs2*$^{-/-}$: 120±11.5 mg/dL) as compared with mice deficient for this gene (*apoE*$^{-/-}$*:* 341.3±17.8 mg/dL, *apoE*$^{-/-}$*Irs2*$^{-/-}$: 330.7±58.5 mg/dL) (Figure 2A). While plasma cholesterol remained essentially unchanged in fat-fed WT mice (129.2±20 mg/dL), all groups of genetically-modified animals developed hypercholesterolemia, which reached highest values in *apoE*$^{-/-}$ (664±28 mg/dL) and *apoE*$^{-/-}$*Irs2*$^{-/-}$ (600.3±45.3 mg/dL) as compared with *Irs2*$^{-/-}$ (278.0±24.6 mg/dL) mice. This rise in total cholesterol was mostly produced in the non-HDL cholesterol fraction, which was also lower in WT and *Irs2*$^{-/-}$ when compared to *apoE*$^{-/-}$ and *Irs2*$^{-/-}$*apoE*$^{-/-}$ mice under either dietary regimen (Figure 2A). *Irs2*$^{-/-}$ mice fed standard chow exhibited the highest level of HDL-cholesterol, which decreased upon high-fat feeding to reach levels similar to those seen in WT, *apoE*$^{-/-}$ and *Irs2*$^{-/-}$*apoE*$^{-/-}$ mice. Only *apoE*$^{-/-}$ mice exhibited a modest but statistically significant increase in plasma triglycerides upon high-fat feeding (Figure 2B).

4.2. Accelerated atherosclerosis in fat-fed *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice can be predicted by high level of circulating insulin

We next examined in the four groups of mice fed the high-fat diet for 4 weeks the development of atherosclerosis in *en face* aortic preparations stained with Oil Red O (Figure 3A). Upon gross macroscopic examination, atherosclerotic lesions were largely absent within the aortic arch and thoracic aorta of WT and *Irs2*$^{-/-}$ mice. In contrast, *apoE*$^{-/-}$ mice exhibited clearly visible atheromas, particularly within the aortic arch region, consistent with numerous studies in this mouse model. These observations were corroborated by computer-

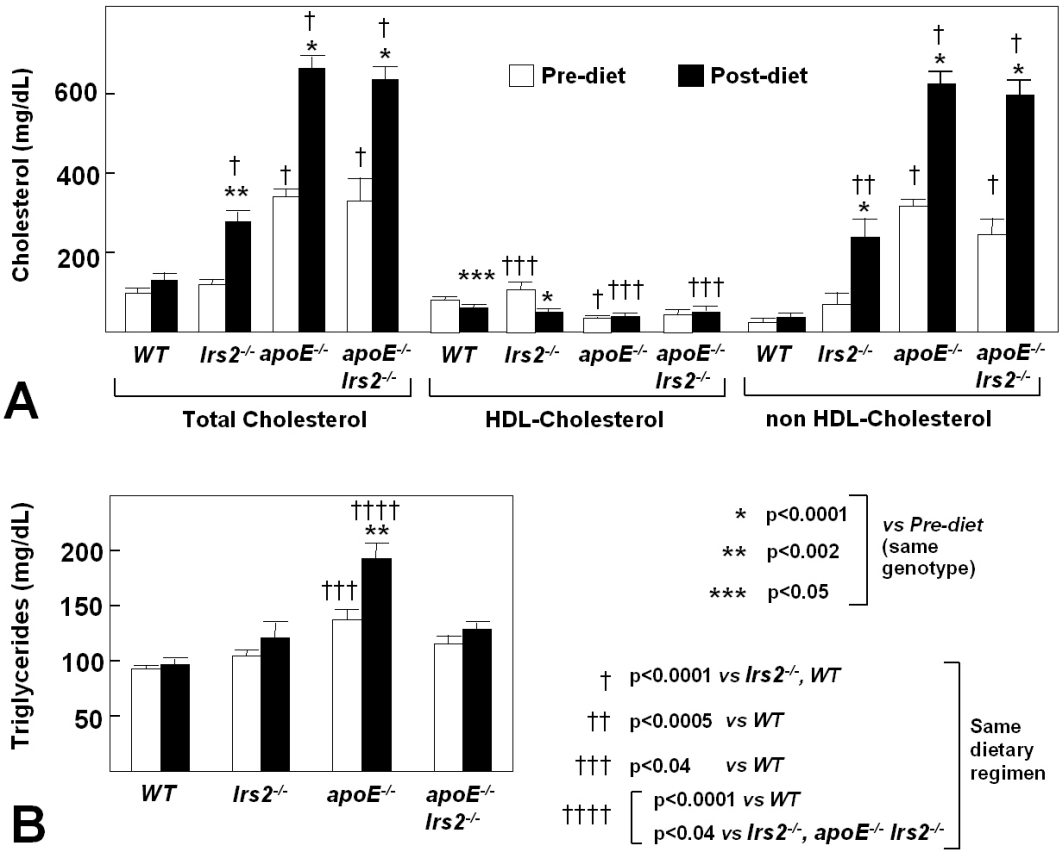

Figure 2. Analysis of plasma lipid levels. Mice were fed control chow until the age of 2 months (white bars) and then were switched to the atherogenic diet for 4 weeks (black bars). Total cholesterol, HDL-cholesterol, non-HDL cholesterol and triglycerides were quantified in WT (n=14, 6 females, 8 males), *Irs2*$^{-/-}$ (n=8, 4 females, 4 males), *apoE*$^{-/-}$ (n=18, 7 females, 11 males) and *apoE*$^{-/-}$*Irs2*$^{-/-}$ (n=6, 3 females, 3 males) mice. Results represent the mean±SE. For simplicity, only relevant statistical comparisons are shown.

assisted planimetric analysis which revealed a significant increase in aortic arch atherosclerotic lesion size in *apoE*$^{-/-}$ *Irs2*$^{-/-}$ versus *apoE*$^{-/-}$ mice ($p<0.01$), a tendency that was also noted within the thoracic aorta. Furthermore, quantification of the intima-to-media ratio (I/M) in transverse sections through the aortic sinus, a highly atherogenic vascular bed in the mouse, also demonstrated accelerated atherosclerosis in *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice ($p=0.050$ versus *apoE*$^{-/-}$) (Figure 4A).

We carried out regression analysis to ascertain whether aortic arch atherosclerosis development correlated with metabolic parameters. As revealed by the *F*-test, atheroma size and circulating levels of glucose and cholesterol did not correlate in fat-fed *apoE*$^{-/-}$ and *apoE*$^{-/-}$ *Irs2*$^{-/-}$ mice (data not shown). However, this test revealed a significant direct correlation between plasma insulin level and aortic arch atherosclerotic lesion size in *apoE*$^{-/-}$*Irs2*$^{-/-}$ but not in *apoE*$^{-/-}$ mice (Figure 5).

4.3. Neointimal VSMC and macrophage content is not altered in *apoE*$^{-/-}$*Irs2*$^{-/-}$ versus *apoE*$^{-/-}$ mice

We next carried out immunohistochemical studies in aortic sinus cross-sections to identify neointimal macrophages and VSMCs, using antibodies against Mac3 (Figure 4B) and SM alpha-actin (Figure 4C), respectively. These studies revealed that atheromas in *apoE*$^{-/-}$ and *apoE*$^{-/-}$ *Irs2*$^{-/-}$ mice consisted of fatty streaks with a similar cellular composition, with predominance of macrophages and lower VSMC content. Although the differences versus *apoE*$^{-/-}$ and *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice did not reach statistical significance, the very small lesions in *Irs2*$^{-/-}$ mice had a tendency towards reduced macrophage content, possibly due to the presence of undifferentiated monocytes which do not exhibit Mac3 immunoreactivity.

5. DISCUSSION

In this study using genetically-modified mice, we provide evidence that severe hypercholesterolemia imposed on IRS2 deficiency (>600 mg/dL, fat-fed *apoE*$^{-/-}$ *Irs2*$^{-/-}$ mouse) aggravates atherosclerosis. These findings are consistent with recently published studies demonstrating that partial (16) or global (17) *Irs2* inactivation accelerate atheroma development in *apoE*$^{-/-}$ mice, and also contribute the following novel insight into the mechanisms that link IRS2 deficiency to atherosclerosis: 1) we found that atherosclerotic lesions

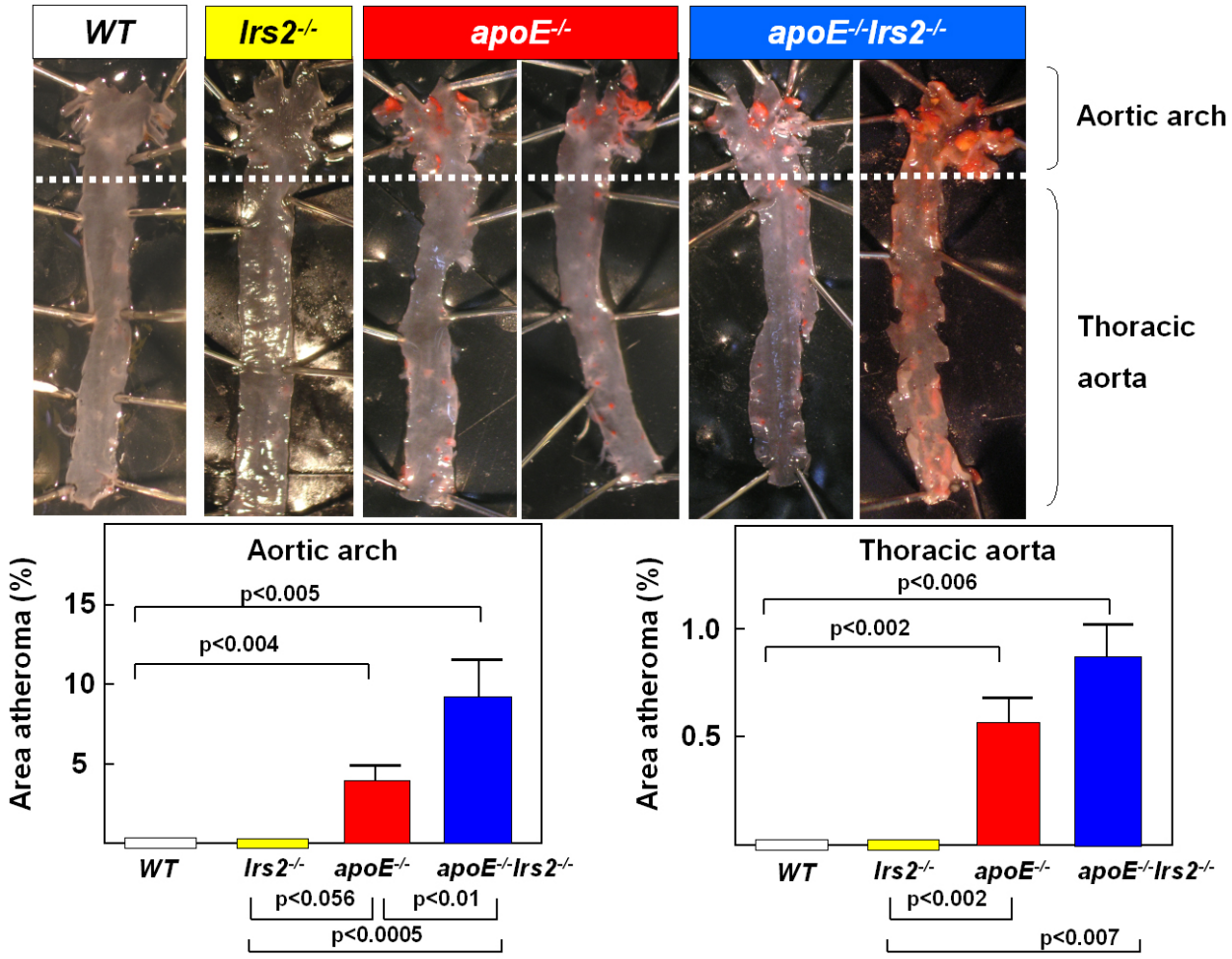

Figure 3. *Irs2*$^{-/-}$*apoE*$^{-/-}$ mice exhibit accelerated aortic atherosclerosis. Mice were sacrificed after 4 weeks of high-fat feeding and the aorta was processed for *en face* Oil-red O staining to quantify atherosclerotic lesion formation in the aortic arch and thoracic aorta by a researcher who was blinded to genotype. WT: n=14 (6 females, 8 males), *Irs2*$^{-/-}$: n=8 (4 females, 4 males), *apoE*$^{-/-}$: n=16 (7 females, 9 males), *apoE*$^{-/-}$*Irs2*$^{-/-}$: n=7 (3 females, 4 males). Results represent the mean±SE.

were largely absent in fat-fed hyperinsulinemic, hyperglycaemic *Irs2*$^{-/-}$ mice which presented more modest levels of plasma cholesterol (~280 mg/dL). Thus, in the absence of severe hypercholesterolemia, *Irs2* disruption is not sufficient to promote atherosclerosis in the mouse. This is in marked contrast with the observation that normocholesterolemic (~70 mg/dL) *Irs2*$^{-/-}$ mice display accelerated neointimal lesion development upon mechanical injury of the femoral artery (18); 2) our regression analysis indicates that atherosclerosis predisposition in *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice can be predicted by circulating insulin levels; 3) in contrast, plasma glucose levels did not predict the burden of atherosclerosis in *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice, and atheromas were largely absent in fat-fed hyperglycaemic *Irs2*$^{-/-}$ mice. These findings highlight an important difference between type 1 and type 2 diabetes, since sustained hyperglycaemia alone without secondary lipid abnormalities is sufficient to accelerate murine atherosclerosis in the setting of type 1 diabetes (19,20). Further evidence that hyperglycaemia is not a predisposing factors for type 2 diabetes-induced atherosclerosis is provided by observations in various obesity-related models of diabetes and atherosclerosis (21-24); 4) we found that acceleration of atherosclerosis in *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice is not accompanied by changes in neointimal VSMC and macrophage content in early fatty streaks. Due to the high mortality rate associated with development of diabetes under conditions of IRS2 deficiency, it was impossible to evaluate whether this genetic manipulation may affect lesion composition at advanced disease states (eg., old fat-fed *apoE*$^{-/-}$*Irs2*$^{-/-}$ versus *apoE*$^{-/-}$ mice), and to investigate the effects on atherosclerosis of long-term exposure of *Irs2*$^{-/-}$ mice to high-fat diet.

Surprisingly, we observed that fasting hyperglycaemia and glucose intolerance was ameliorated in *apoE*$^{-/-}$*Irs2*$^{-/-}$ in comparison with *Irs2*$^{-/-}$ mice, which did not appear to be related to a preservation of beta-cell mass (data not shown). Previous studies have shown that *Irs2* disruption upregulates the expression of lipogenic enzymes in the liver (25), which in turn enhances hepatic lipid uptake as well as lipogenesis. Given that apoE is the major

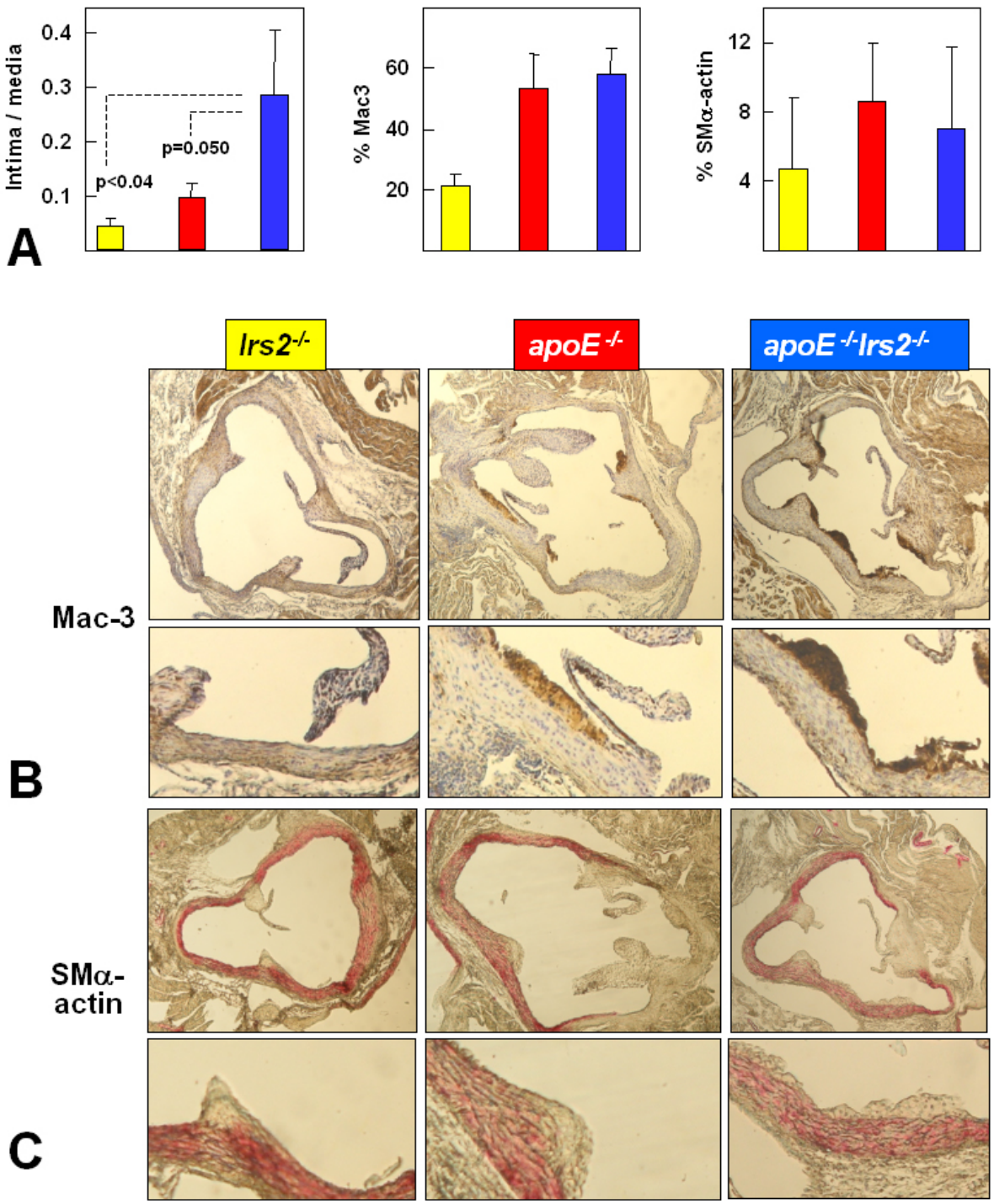

Figure 4. Immunohistochemical characterization of atherosclerotic lesions. Mice were maintained for 4 weeks on the atehrogenic diet and atherosclerosis in cross-sections through the aortic sinus was quantified by an operator who was blinded to genotype. Data for *Irs2*$^{-/-}$, *apoE*$^{-/-}$ and *apoE*$^{-/-}$*Irs2*$^{-/-}$ are shown in yellow, red and blue, respectively. (A) Quantification of the intima-to-media ratio (for each mouse, average from 3 independent cross-sections), neointimal Mac3-immunoreactive macrophages and neointimal SM alpha-actin-immunoreactive VSMCs. Results represent the mean±SE of 4 *Irs2*$^{-/-}$, 8 *apoE*$^{-/-}$ and 5 *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice. Representative images of Mac 3 (B) and SM alpha-actin (C) immunostaining are shown. The lower photomicrographs correspond to enlarged areas of the specimens shown on top.

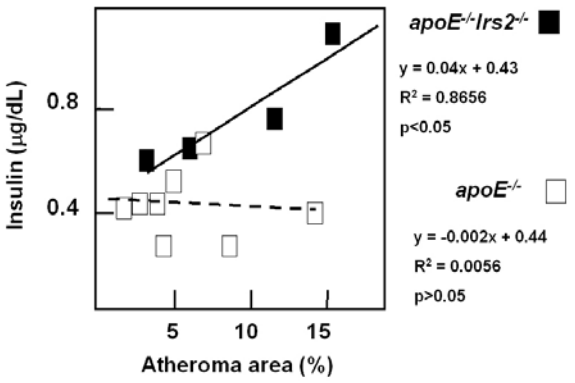

Figure 5. Plasma insulin levels predict the burden of atherosclerosis in *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice. Regression analysis of atheroma size within the aortic arch as a function of plasma insulin levels. Each point represents one animal. The *F*-test revealed a statistically significant correlation between plasma insulin levels and atheroma size only in *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice.

ligand for hepatic lipoprotein and lipid clearance, the possibility exists that reduced plasma glucose in *apoE*$^{-/-}$ *Irs2*$^{-/-}$ versus *Irs2*$^{-/-}$ mice may reflect a compensatory increase in lipogenesis to maintain lipid homeostasis.

Studies in mice (26) and humans (27,28) have linked insulin signalling alterations with increased lipid accumulation in macrophages and foam cell formation due to enhanced CD36 receptor expression. Consistent with this, transplant of insulin receptor (IR)-null bone marrow into irradiated low-density lipoprotein receptor-null mice (*Ldlr*$^{-/-}$) increased lipid uptake through the upregulation of CD36 and SRA, thereby aggravating atherosclerosis (29). In contrast, Baumgartl and co-workers reported that macrophage-specific deficiency of either IR or IRS2 ameliorates atherosclerosis development in *apoE*$^{-/-}$ mice (17). Although the reasons for this apparent discrepancy are unclear, it appears that modulation of glucose and insulin signalling in macrophages plays a direct role in lipid deposition and foam cell formation in the vessel wall.

Despite the presence of insulin resistance and maintenance on a high-fat diet, *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice did not develop obesity during our study (Figure 1C). In contrast, mice homozygous for the diabetes (*Lepr*$^{db/db}$) and obesity (*Lep*$^{ob/ob}$) mutations display phenotypes of obesity-induced diabetes (30). When either of these models is combined with deficiency for apoE, atherosclerosis is enhanced in double mutants fed normal rodent chow and this tendency is further aggravated by high-fat regimen (21,23,24). Atherosclerosis is also increased in *Ldlr*$^{-/-}$*Lep*$^{ob/ob}$ mice, although to a lesser extent than in *apoE*$^{-/-}$*Lep*$^{ob/ob}$ mice (22,23). Unlike these obese/diabetic models, atherosclerosis in *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice was not associated with either obesity or aggravated dyslipidemia. However, one common feature of all these models is hyperinsulinemia, suggesting that plasma insulin levels predict atherosclerotic lesion burden. However, it should be noted that insulin resistance and hyperinsulinemia do not enhance atherosclerosis in *Ldlr*$^{-/-}$ mice under conditions of very high hypercholesterolemia (up to 2000 mg/dL) (31). Thus, these different murine models offer excellent tools for unraveling distinct molecular links between atherosclerosis development and various risk factors associated with diabetes and metabolic syndrome.

In conclusion, our findings suggest that hyperglycaemia and hyperinsulinemia are not sufficient to promote atherosclerosis under conditions of mild hypercholesterolemia (~280 mg/dL, fat-fed *Irs2*$^{-/-}$ mice). However, when combined with severe hypercholesterolemia (>600 mg/dL, fat-fed *apoE*$^{-/-}$*Irs2*$^{-/-}$ mice), the systemic insulin resistance produced by the absence of IRS2 facilitates the development of atherosclerosis in a manner that can be predicted by circulating insulin levels. Future studies are warranted to determine whether IRS2 dysfunction contributes to human atherosclerosis in individuals with hyperinsulinemia but without overt hyperglycaemia, and in type 2 diabetic patients with or without metabolic syndrome.

6. ACKNOWLEDGEMENTS

We thank M. J. Andrés-Manzano for help with immunohistochemistry and for the preparation of figures. This work was supported by grants from the Spanish Ministry of Education and Science and the European Regional Development Fund (SAF2004-03057), from Instituto de Salud Carlos III (Red de Centros RECAVA, C03/01; Red de Grupos G03-212), and from the European Union (Marie Curie fellowship MEIF-CT-2005-024393). M.V.-C is the recipient of a fellowship from the Regional Government of Valencia.

7. REFERENCES

1. Beckman J. A., M. A. Creager & P. Libby: Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *Jama* 287, 2570-2581 (2002)
2. Gerstein H. C. & S. Yusuf: Dysglycaemia and risk of cardiovascular disease. *Lancet* 347, 949-950 (1996)
3. Schwartz S. M. & K. E. Bornfeldt: How does diabetes accelerate atherosclerotic plaque rupture and arterial occlusion? *Front Biosci* 8, s1371-1383 (2003)
4. Moreno P. R. & V. Fuster: New aspects in the pathogenesis of diabetic atherothrombosis. *J Am Coll Cardiol* 44, 2293-2300 (2004)
5. Ginsberg H. N.: Insulin resistance and cardiovascular disease. *J Clin Invest* 106, 453-458 (2000)
6. Withers D. J., J. S. Gutierrez, H. Towery, D. J. Burks, J. M. Ren, S. Previs, Y. Zhang, D. Bernal, S. Pons, G. I. Shulman, S. Bonner-Weir & M. F. White: Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* 391, 900-904 (1998)
7. Withers D. J., D. J. Burks, H. H. Towery, S. L. Altamuro, C. L. Flint & M. F. White: Irs-2 coordinates Igf-1 receptor-mediated beta-cell development and peripheral insulin signalling. *Nat Genet* 23, 32-40 (1999)
8. Kubota N., K. Tobe, Y. Terauchi, K. Eto, T. Yamauchi, R. Suzuki, Y. Tsubamoto, K. Komeda, R. Nakano, H. Miki, S. Satoh, H. Sekihara, S. Sciacchitano, M. Lesniak, S. Aizawa,

R. Nagai, S. Kimura, Y. Akanuma, S. I. Taylor & T. Kadowaki: Disruption of insulin receptor substrate 2 causes type 2 diabetes because of liver insulin resistance and lack of compensatory beta-cell hyperplasia. *Diabetes* 49, 1880-1889 (2000)
9. Burks D. J., J. F. de Mora, M. Schubert, D. J. Withers, M. G. Myers, H. H. Towery, S. L. Altamuro, C. L. Flint & M. F. White: IRS-2 pathways integrate female reproduction and energy homeostasis. *Nature* 407, 377-382 (2000)
10. Lin X., A. Taguchi, S. Park, J. A. Kushner, F. Li, Y. Li & M. F. White: Dysregulation of insulin receptor substrate 2 in beta cells and brain causes obesity and diabetes. *J Clin Invest* 114, 908-916 (2004)
11. Nandi A., Y. Kitamura, C. R. Kahn & D. Accili: Mouse models of insulin resistance. *Physiol Rev* 84, 623-647 (2004)
12. Hennige A. M., D. J. Burks, U. Ozcan, R. N. Kulkarni, J. Ye, S. Park, M. Schubert, T. L. Fisher, M. A. Dow, R. Leshan, M. Zakaria, M. Mossa-Basha & M. F. White: Upregulation of insulin receptor substrate-2 in pancreatic beta cells prevents diabetes. *J Clin Invest* 112, 1521-1532 (2003)
13. Meir K. S. & E. Leitersdorf: Atherosclerosis in the apolipoprotein E-deficient mouse: a decade of progress. *Arterioscler Thromb Vasc Biol* 24, 1006-1014 (2004)
14. González-Navarro H., Z. Nong, M. J. Amar, R. D. Shamburek, J. Najib-Fruchart, B. J. Paigen, H. B. Brewer, Jr. & S. Santamarina-Fojo: The ligand-binding function of hepatic lipase modulates the development of atherosclerosis in transgenic mice. *J Biol Chem* 279, 45312-45321 (2004)
15. Díez-Juan A. & V. Andrés: The growth suppressor $p27^{Kip1}$ protects against diet-induced atherosclerosis. *Faseb J* 15, 1989-1995 (2001)
16. Clough M. H., D. J. Schneider, B. E. Sobel, M. F. White, M. P. Wadsworth & D. J. Taatjes: Attenuation of accumulation of neointimal lipid by pioglitazone in mice genetically deficient in insulin receptor substrate-2 and apolipoprotein E. *J Histochem Cytochem* 53, 603-610 (2005)
17. Baumgartl J., S. Baudler, M. Scherner, V. Babaev, L. Makowski, J. Suttles, M. McDuffie, S. Fazio, C. R. Kahn, G. S. Hotamisligil, W. Krone, M. Linton & J. C. Bruning: Myeloid lineage cell-restricted insulin resistance protects apolipoproteinE-deficient mice against atherosclerosis. *Cell Metab* 3, 247-256 (2006)
18. Kubota T., N. Kubota, M. Moroi, Y. Terauchi, T. Kobayashi, K. Kamata, R. Suzuki, K. Tobe, A. Namiki, S. Aizawa, R. Nagai, T. Kadowaki & T. Yamaguchi: Lack of insulin receptor substrate-2 causes progressive neointima formation in response to vessel injury. *Circulation* 107, 3073-3080 (2003)
19. Kunjathoor V. V., D. L. Wilson & R. C. LeBoeuf: Increased atherosclerosis in streptozotocin-induced diabetic mice. *J Clin Invest* 97, 1767-1773 (1996)
20. Renard C. B., F. Kramer, F. Johansson, N. Lamharzi, L. R. Tannock, M. G. von Herrath, A. Chait & K. E. Bornfeldt: Diabetes and diabetes-associated lipid abnormalities have distinct effects on initiation and progression of atherosclerotic lesions. *J Clin Invest* 114, 659-668 (2004)
21. Wu K. K., T. J. Wu, J. Chin, L. J. Mitnaul, M. Hernandez, T. Q. Cai, N. Ren, M. G. Waters, S. D. Wright & K. Cheng: Increased hypercholesterolemia and atherosclerosis in mice lacking both ApoE and leptin receptor. *Atherosclerosis* 181, 251-259 (2005)
22. Hasty A. H., H. Shimano, J. Osuga, I. Namatame, A. Takahashi, N. Yahagi, S. Perrey, Y. Iizuka, Y. Tamura, M. Amemiya-Kudo, T. Yoshikawa, H. Okazaki, K. Ohashi, K. Harada, T. Matsuzaka, H. Sone, T. Gotoda, R. Nagai, S. Ishibashi & N. Yamada: Severe hypercholesterolemia, hypertriglyceridemia, and atherosclerosis in mice lacking both leptin and the low density lipoprotein receptor. *J Biol Chem* 276, 37402-37408 (2001)
23. Gruen M. L., V. Saraswathi, A. M. Nuotio-Antar, M. R. Plummer, K. R. Coenen & A. H. Hasty: Plasma insulin levels predict atherosclerotic lesion burden in obese hyperlipidemic mice. *Atherosclerosis* (2005)
24. Wendt T., E. Harja, L. Bucciarelli, W. Qu, Y. Lu, L. L. Rong, D. G. Jenkins, G. Stein, A. M. Schmidt & S. F. Yan: RAGE modulates vascular inflammation and atherosclerosis in a murine model of type 2 diabetes. *Atherosclerosis* 185, 70-77 (2006)
25. Taniguchi C. M., K. Ueki & R. Kahn: Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism. *J Clin Invest* 115, 718-727 (2005)
26. Liang C. P., S. Han, H. Okamoto, R. Carnemolla, I. Tabas, D. Accili & A. R. Tall: Increased CD36 protein as a response to defective insulin signaling in macrophages. *J Clin Invest* 113, 764-773 (2004)
27. Griffin E., A. Re, N. Hamel, C. Fu, H. Bush, T. McCaffrey & A. S. Asch: A link between diabetes and atherosclerosis: Glucose regulates expression of CD36 at the level of translation. *Nat Med* 7, 840-846 (2001)
28. Sampson M. J., I. R. Davies, S. Braschi, K. Ivory & D. A. Hughes: Increased expression of a scavenger receptor (CD36) in monocytes from subjects with Type 2 diabetes. *Atherosclerosis* 167, 129-134 (2003)
29. Han S., C. P. Liang, T. Devries-Seimon, M. Ranalletta, C. L. Welch, K. Collins-Fletcher, D. Accili, I. Tabas & A. R. Tall: Macrophage insulin receptor deficiency increases ER stress-induced apoptosis and necrotic core formation in advanced atherosclerotic lesions. *Cell Metab* 3, 257-266 (2006)
30. Coleman D. L.: Obesity genes: beneficial effects in heterozygous mice. *Science* 203, 663-665 (1979)
31. Merat S., F. Casanada, M. Sutphin, W. Palinski & P. D. Reaven: Western-type diets induce insulin resistance and hyperinsulinemia in LDL receptor-deficient mice but do not increase aortic atherosclerosis compared with normoinsulinemic mice in which similar plasma cholesterol levels are achieved by a fructose-rich diet. *Arterioscler Thromb Vasc Biol* 19, 1223-1230 (1999)

Key Words: Atherosclerosis, Diabetes, Hypercholesterolemia, Insulin Receptor Substrate 2, Insulin, Insulin Resistance, Genetically-Modified Mice

Send correspondence to: Dr Vicente Andrés, Vascular Biology Laboratory, Department of Molecular and Cellular Pathology and Therapy, Instituto de Biomedicina de Valencia (IBV-CSIC), Jaime Roig 11, 46010 Valencia, Spain, Tel: +34-963391752, Fax: +34-963391751, E-mail: vandres@ibv.csic.es