The Proinflammatory Mediator CD40 Ligand Is Increased in the Metabolic Syndrome and Modulated by Adiponectin

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Objectives: We hypothesized that the CD40/CD40 ligand (CD40L) system is up-regulated in the metabolic syndrome (MS) and modulated by adiponectin (AN). The objectives were: 1) to compare plasma and monocyte CD40L in patients with MS and controls and its association with clinical and biochemical parameters, 2) to investigate platelets as a source of soluble CD40L (sCD40L), and 3) to analyze the effects of AN on CD40/CD40L.

Methods: Plasma sCD40L and AN were measured in 246 controls and 128 patients with MS by ELISA. Monocyte CD40/CD40L expression and platelet CD40L content and release were compared in patients with MS and controls. Monocytes and endothelial cells were cultured with AN and CD40/CD40L expression determined by real-time RT-PCR and Western blotting.

Results: Patients with MS had higher sCD40L and lower AN levels than controls (0.89 ± 0.1 vs. 0.76 ± 0.07 ng/ml and 10.10 ± 0.65 vs. 12.99 ± 0.80 μg/ml, P < 0.05). Monocyte CD40/CD40L expression was higher (P < 0.05) in patients than controls (CD40: 1.31 ± 0.31 vs. 0.80 ± 0.14 arbitrary units; CD40L: 1.24 ± 0.85 vs. 0.43 ± 0.14 pg/μg protein). No differences were observed on CD40L content between resting platelets from patients with MS and controls (7.7 ± 3.5 vs. 7.2 ± 2.2 pg/μg protein). Stimulated platelets from patients with the MS released more (P < 0.05) sCD40L than controls (582 ± 141 vs. 334 ± 60% change vs. nonstimulated platelets). AN reduced CD40L mRNA and protein expression in monocytes from MS patients and endothelial cells.

Conclusions: The enhanced sCD40L and cellular CD40L expression in the MS suggests that CD40L is of pathophysiological relevance in MS. Also, a new antiinflammatory effect of AN is described through the modulation of the CD40/CD40L system. (J Clin Endocrinol Metab 93: 2319–2327, 2008)

The metabolic syndrome (MS) is characterized by a general proinflammatory and prothrombotic state that interacts synergistically causing or accelerating the progression of atherosclerosis (1, 2).

CD40 ligand (CD40L) and its receptor CD40 are expressed in a wide variety of cells including vascular cells (3). There is in vitro (4) and in vivo (5) evidence of the participation of CD40-CD40L interactions in atherothrombosis. In addition to the cell-associated form, CD40L also exists in a circulating soluble form (sCD40L). In humans, enhanced plasma sCD40L levels predict primary (6) and secondary (7) cardiovascular (CV) events and correlate with composition characteristics of the atheroma plaque (8). Furthermore, sCD40L plasma levels associate with other known CV risk factors, such as diabetes (9) and hypercholesterolemia (10). In these two conditions, there is increased expression of both the cell-associated and soluble form. MS is also independently associated with elevated sCD40L (11–13). However, surface CD40 and CD40L expression on circulating cells of patients with MS and the possible sources that explain the

Abbreviations: AN, Adiponectin; BMI, body mass index; CD40L, CD40 ligand; CRP, C-reactive protein; CV, cardiovascular; DBP, diastolic blood pressure; HDL, high-density lipoprotein; HOMA, homeostasis model assessment; HUVEC, human umbilical cord; ICAM, intercellular adhesion molecule; IMT, intima-media thickness; MS, metabolic syndrome; sCD40L, soluble CD40L; SBP, systolic blood pressure; TG, triglycerides; VCAM, vascular cell adhesion molecule.
overexpression of sCD40L in this syndrome have not been explored.

Adipose tissue, in excess in the MS, releases bioactive molecules named adipokines, such as adiponectin (AN) and others. Reduced serum AN concentrations correlate with obesity, insulin resistance, and type 2 diabetes (14). In addition to its metabolic actions, AN also improves endothelial function and vascular homeostasis by binding to its receptors, AdipoR1 and AdipoR2, on monocytes, smooth muscle cells, and endothelial cells (15–17). These cell types are involved at all stages of atherosclerosis and mediate inflammatory actions through the CD40/CD40L dyad. However, the interplay between AN and CD40L has not been explored.

Thus, we hypothesized that the CD40/CD40L system is upregulated in the MS and could be modulated by AN. The objectives were: 1) to compare plasma and monocyte CD40L expression in patients with MS with controls and its association with clinical and biochemical parameters, 2) to investigate platelets as a source of circulating sCD40L levels in the MS, and 3) to analyze the possible antiinflammatory effects of AN on the CD40/CD40L system on monocytes and endothelial cells.

Subjects and Methods

Study population

The study was performed in 246 subjects without MS (73% men, 54 ± 11 yr) and 128 patients with MS (91% men, 56 ± 11 yr) attending the Cardiovascular Risk Area of the University Clinic of Navarra for a general check-up. Subjects were free from clinically apparent atherosclerotic disease based on absence of history of coronary disease, stroke, or peripheral artery disease and normal electrocardiogram. Exclusion criteria were impaired renal or liver function, arteritis, and connective tissue diseases. None of the subjects studied presented any inflammatory disease or condition associated with alterations in plasma sCD40L levels. The local committee on human research approved the study, performed in accordance with the Declaration of Helsinki, and all participants gave written informed consent.

All participants underwent a complete medical examination and anthropometric measurements. Blood pressure was measured using a mercury sphygmomanometer. The average of two measurements was considered.

MS was diagnosed according to the National Cholesterol Education Program-Adult Treatment Panel III guidelines (18, 19) with modification of waist criterion into body mass index (BMI; ≥ 30 kg/m²).

Clinical analyses

All subjects underwent ultrasonography of the common carotid arteries performed with a 5- to 12-MHz linear-array transducer (ATL 500 HDI, Bothell, Washington). The measurement of intima-media thickness (IMT) was made 1 cm proximal to the carotid bulb of each carotid artery at plaque-free sites. From each individual, the IMT was determined as the average of near- and far-wall measurements of each carotid artery. In addition, the presence or absence of atheromatous plaques was determined.

Biochemical analyses

Serum and plasma were collected in Vacutainer tubes. Fasting serum glucose, cholesterol, triglycerides (TG), and high-density lipoprotein (HDL) cholesterol were measured by standard laboratory techniques. Insulin levels were determined by chemiluminescence in the Immulite 2000 (Diagnostic Products Corp., Los Angeles, CA). Plasma levels of IL-12, IL-18, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and C-reactive protein (CRP) were measured by ELISA (R&D Systems, Minneapolis, MN). Fibrinogen activity was determined by coagulation assay (Dade Behring, Marburg, Germany).

The homeostasis model assessment (HOMA) of insulin resistance

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MAP, Mean arterial pressure; LDL, low-density lipoprotein.

* P < 0.001 vs. group with no factors.

** P < 0.001 vs. group with one to two factors.
was calculated as insulin (microunits per milliliter) × glucose (millimoles per liter)/22.5.

sCD40L in serum and platelet supernatants or lysates (10 μg) was determined by ELISA (BenderMed Systems, Vienna, Austria), as previously described (9). The intraassay variation was less than 10%. AN concentration was measured by ELISA (R&D Systems). Intraassay variation was less than 15%.

Reagents

RPMI 1640, penicillin/streptomycin mixture, fetal calf serum, PBS, trypsin-EDTA, endothelial cell serum-free medium, and l-glutamine were purchased from Life Technologies, Inc. (Paisley, UK). MEM199 and Hank’s balanced salt solution were purchased from BioWhittaker (Verviers, Belgium) and collagenase A from Clostridium histolyticum and protease inhibitors cocktail from Roche Molecular Biochemicals (Mannheim, Germany). Recombinant TNF-α and bovine gelatin were purchased from Sigma (St. Louis, MO) and recombiant human adiponectin/adipolein from PeproTech EC (London, UK).

Monocyte isolation and stimulation

For in vitro studies, cells from subjects with (three to five factors, n = 20) or without MS (none to two factors, n = 20) were collected to increase the statistical power. Lymphomononuclear cells were obtained from peripheral blood as described by Boyum (20). Cells were incubated in RPMI 1640 medium with 10% heat-decomplemented fetal calf serum, 2 mmol/liter l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (37°C, 5% CO2). Human monocytes were isolated by adherence to plastic surface. CD40/CD40L expression were determined by real-time RT-PCR and Western blotting or ELISA.

Platelet isolation

Platelets were isolated from patients with MS (n = 15) and controls (n = 15) by layering citrated blood over a density barrier [Optiprep (Fresenius; Oslo, Norway); 350 g, 15 min, 20°C]. The platelet-containing band was resuspended in 140 mmol/liter NaCl, 3 mmol/liter Tris (pH 8), 150 mmol/liter NaCl, 10% glycerol, 1% Triton X-100, 2 mmol/liter EDTA, protease inhibitors cocktail, and bovine gelatin were purchased from Sigma (St. Louis, MO) and recombiant human adiponectin/adipolein from PeproTech EC (London, UK).

Platelet isolation kit (QIAGEN, Valencia, CA). Reverse transcriptase was performed with 250 ng total RNA. Real-time RT-PCR was performed with 50 ng cDNA in an ABI PRISM7000 sequence detection system using specific TaqMan MGB probes (Applied Biosystems, Foster City, CA). Constitutive 18S ribosomal RNA was used as endogenous control.

For each experimental condition, the mean quantity of target gene and endogenous control was obtained from a standard curve (representing cycle threshold values as the log amount of starting material) in triplicate. The sd among triplicates was less than 0.2. The mean quantity value of the target gene was divided by the mean value of the endogenous control to obtain a normalized mean quantity per sample.

Western blotting

Monocytes or endothelial cell extracts were harvested in lysis buffer [20 mmol/liter Tris (pH 8), 150 mmol/liter NaCl, 10% glycerol, 1% Triton X-100, 2 mmol/liter EDTA, protease inhibitors cocktail] and protein concentration assessed by the Bradford method (Bio-Rad, Hercules, CA). Fifteen micrograms of protein were fractionated on 12% polyacrylamide gels and transferred into nitrocellulose membranes. Membranes were blocked (5% nonfat dry milk in 0.05% Tween-20 PBS) and incubated with a mouse monoclonal anti-human CD40L (1:1000; BenderMedSystems) or rabbit polyclonal antihuman CD40 (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibody was detected by peroxidase-conjugated secondary antibody and visualized using the ECL-Plus chemiluminescence detection system (both Amersham Biosciences, Piscataway, NJ).

Endothelial cells isolation and stimulation

Endothelial cells were isolated from human umbilical cords (HUVECs) as described by Jaffe et al. (21) and incubated in MEM199 containing 20% human serum (37°C, 5% CO2) until confluence.

Twenty-four hours before stimulation, cultures were washed with Hank’s balanced salt solution and fresh serum-free medium with antibiotics. Cultures were stimulated with AN (30 μg/ml) in the presence of a pretreatment with TNF-α (50 ng/ml), a known inducer of CD40L expression (22). Additional nonstimulated or stimulated with only TNF-α (50 ng/ml) cultures were maintained as negative controls and positive controls, respectively. Gene (4 h) and protein (24 h) CD40/CD40L expression was determined by real-time RT-PCR and Western blotting. As a control of the stimulation, levels of IL-6 and ICAM-1 were measured in culture supernatants by ELISA. A, Patients with more than three risk factors of the MS (n = 128) had higher circulating sCD40L levels than those with one or two (n = 187) or controls (n = 59). B, AN levels decreased with the number of risk factors, compared with controls. Bars represent mean sCD40L or AN concentrations ± SEM. n.s., Nonsignificant. *, P < 0.05.
Statistical analysis

The statistical analysis was performed with SPSS (version 13.0; SPSS, Chicago, IL). The normal distribution of variables was tested with the Shapiro-Wilk test. Differences across groups were compared by ANOVA followed by the Bonferroni post hoc test for normal variables and the Kruskal Wallis for nonnormal variables. The unpaired Mann-Whitney U test was used to assess statistical differences between experimental conditions. Spearman correlation coefficients for continuous variables were also used to assess univariate correlations of sCD40L or AN levels with all variables. Multivariate linear regression analysis was performed to assess the independent relationships between sCD40L levels and other variables after adjusting for other confounding factors. Results are presented as mean ± SEM. P < 0.05 was considered significant.

Results

Human studies

Demographic and clinical characteristics of the study population

To provide more detailed information, subjects were divided into three groups according to the number of risk factors of the MS (none, one to two, three to five). All parameters will be compared with this classification in the following. Demographic and clinical characteristics of the study population are presented in Table 1. Patients with the MS had higher BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), glucose, HOMA, and TG levels than patients without the MS (one to two or no factors, all P < 0.001). HDL-C levels were lower in patients with the MS, compared with the other two groups (both P < 0.001). Also, BMI, SBP, DBP, glucose, HOMA, and TG were higher and HDL-C lower in patients with one to two factors, compared with none.

We next characterized the proinflammatory profile of the studied population. Patients with more than three risk factors of the MS had higher (P < 0.05) levels of IL-12, IL-18, CRP, and fibrinogen than those with one to two and higher CRP and fibrinogen than those with no factors (Table 1). Subjects with three to five factors had higher VCAM-1 (P < 0.05) levels than subjects with none.

Plasma sCD40L and adiponectin levels in patients with MS

Interestingly, patients with more than three factors of the MS had higher sCD40L levels than those with one to two or none

FIG. 2. CD40 and CD40L expression in monocytes. Monocytes were obtained from healthy donors (n = 20) and patients with the MS (n = 20) by Ficoll gradient and mRNA and protein isolated. CD40 and CD40L expression were determined by real-time RT-PCR (A) and Western blotting (CD40) or ELISA (CD40L) (B). Bars represent mean ± SEM. * P < 0.05.
(0.88 ± 0.1 vs. 0.79 ± 0.08 or 0.65 ± 0.12 ng/ml; all P < 0.05; Fig. 1A). On the contrary, AN levels decreased with the number of risk factors (no factors: 16.5 ± 2.0 μg/ml; one to two factors: 11.98 ± 0.82 μg/ml; three to five factors: 10.10 ± 0.64 μg/ml; all P < 0.05; Fig. 1B). No significant linear association was found between sCD40L and AN plasma concentrations.

In the entire population, no differences were found in sCD40L levels between hypertensive and normotensive patients, between patients with high and low TGs, or low and high HDL cholesterol. In addition, no linear increase in sCD40L circulating concentrations was found among nonobese, overweight, and obese. However, diabetics had higher sCD40L levels than non-diabetics (0.97 ± 0.88 ng/ml, P < 0.05). Interestingly, the presence of the MS leads to higher sCD40L levels in both the diabetic and nondiabetic groups (1.05 ± 0.31 and 0.92 ± 0.14 ng/ml, P < 0.05), suggesting a synergistic effect of risk factors.

We next explored the association of circulating sCD40L and AN levels with other clinical and biochemical parameters. In the whole population, after adjustment by age and gender, sCD40L concentrations correlated with glucose, total cholesterol, CRP, HOMA (all r = 0.2, P < 0.05) and IL-18 (r = 0.3, P < 0.05). AN levels negatively correlated with HDL cholesterol and TG (r = −0.3 and r = −0.4; P < 0.05).

In patients with the MS, sCD40L levels correlated with IL-18 (r = 0.2, P < 0.05). In subjects with no factors of MS, sCD40L levels correlated with IL-18 (r = 0.2, P < 0.05) and VCAM-1 (r = 0.14, P < 0.05), whereas AN levels associated with fibrinogen (r = −0.2, P < 0.05).

No association was found between sCD40L concentrations and carotid IMT. Also, sCD40L plasma concentrations did not differ between patients with or without atheromatous plaques in carotids (0.82 ± 0.38 vs. 0.78 ± 0.17 ng/ml).

**CD40/CD40L system on circulating cells**

**Monocytes**

CD40/CD40L expression were determined on circulating monocytes by real-time RT-PCR for mRNA and Western blotting (CD40) or ELISA (CD40L) for protein. Although we did not find differences in CD40 mRNA expression, monocytes from patients with MS expressed higher mRNA (P < 0.05) of the proinflammatory mediator CD40L (Fig. 2A) than those from subjects without MS (CD40: 346 ± 62 vs. 394 ± 52 arbitrary units; CD40L: 4.8 ± 0.8 vs. 6.5 ± 1.8 arbitrary units). Both CD40/CD40L protein expression, were significantly higher (P < 0.05) in monocytes obtained from patients, compared with controls (CD40: 1.31 ± 0.31 vs. 0.80 ± 0.14 arbitrary units; CD40L: 1.24 ± 0.85 vs. 0.43 ± 0.14 pg/μg of protein; Fig. 2B).

**Platelets**

More than 95% of the circulating sCD40L derives from platelets as a result of activation by different agonists. Thus, to evaluate possible sources of sCD40L, we examined resting CD40L content and sCD40L release from platelets isolated from patients with MS and controls. No significant differences were observed on CD40L intracellular content between resting platelets derived from patients, compared with those from controls (7.7 ± 3.5 vs. 7.2 ± 2.2 pg/μg of protein; Fig. 3A). Incubation with thrombin induced sCD40L release from platelets, but interestingly, platelets derived from patients with the MS released more (P < 0.05) sCD40L than those obtained from subjects without MS after stimulation (382 ± 141 vs. 334 ± 60% change vs. nonstimulated platelets) (Fig. 3B).

**Effects of adiponectin on vascular cells**

**Monocytes**

Given that monocytes express receptors for AN, we next studied the effect of AN on the CD40/CD40L system in monocytes from patients with the MS. CD40/CD40L expression determined by real-time PCR and Western blotting.

mRNA and protein expression of the receptor CD40 did not significantly change after stimulation with AN, compared with nontreated cells (mRNA: 338 ± 87 vs. 524 ± 21 arbitrary units; protein: 0.6 ± 0.1 vs. 0.8 ± 0.2 arbitrary units; Fig. 4). By contrast, AN reduced (P < 0.05) the mRNA and protein expression
of CD40L, compared with nonstimulated cells (mRNA: 3.6 ± 2.0 vs. 7.2 ± 2.5 arbitrary units; protein: 0.8 ± 0.2 vs. 1.9 ± 0.8 arbitrary units) (Fig. 4).

The secretion of the proinflammatory cytokine IL-6 was inhibited (P < 0.05) by AN, compared with nonstimulated cells (10.04 ± 1.53 vs. 12.03 ± 1.3 pg/ml), whereas the secretion of the antiinflammatory cytokine IL-10 was increased (P < 0.01) by AN, compared with nonstimulated monocytes (645 ± 235 vs. 16 ± 5 ng/ml). AN also decreased the expression of the phospho-nuclear factor-κB, compared with the nonphosphorylated form of this nuclear factor, further supporting that AN has an antiinflammatory effect on human monocytes (data not shown).

**Endothelial cells**

To test whether the antiinflammatory effect of AN extends to other vascular cells, some experiments were performed in endothelial cells. CD40/CD40L expression was induced in HUVECs with TNF-α, and cells were then incubated with AN.

Incubation with AN reduced (all P < 0.05) TNF-induced expression of CD40 and CD40L both at mRNA level (CD40: 187 ± 18 vs. 383 ± 137 arbitrary units; CD40L: 14.9 ± 0.1 vs. 32.1 ± 2.7 arbitrary units; Fig. 5A) and protein level (CD40: 0.93 ± 0.03 vs. 1.53 ± 0.01 arbitrary units; CD40L: 0.44 ± 0.13 vs. 3.0 ± 0.04 arbitrary units; Fig. 5B). AN alone had no effect on CD40 or CD40L expression in nonstimulated cells (data not shown).

Finally, AN also inhibited (P < 0.05) IL-6 (60.79 ± 0.00 vs. 97.43 ± 34.65 pg/ml) and ICAM-1 (448 ± 94 vs. 1910 ± 779 ng/ml) secretion.

**Discussion**

The main findings of the present study are: 1) sCD40L levels are increased in patients with MS, compared with controls; 2) circulating monocytes of patients with MS express more CD40 and CD40L than those from controls; 3) platelets from patients with MS release more sCD40L than those from controls after stimulation; and 4) AN decreases CD40L expression in monocytes and endothelial cells.

**Up-regulation of the CD40/CD40L system in the MS**

The MS, with a prevalence of 25% confers increased risk for CV events (1, 23). Inflammation is important precursor of the MS emerging as a predictor of CV disease (24). Our study confirms previous findings of elevated levels of CRP and fibrinogen (25) and expands the characterization of the inflammation in the MS to other inflammatory cytokines and adhesion molecules such as IL-12, IL-18, and VCAM-1. But importantly, levels of the proinflammatory mediator sCD40L increased with the number of risk factors. Some authors have also described an independent association of the MS with elevated sCD40L levels (11–13). However, it is unknown whether enhanced levels could account for increased incidence of CV events in this syndrome despite the fact that several studies have shown that sCD40L can be used as a biomarker for risk stratification (6, 7).

A novel finding of this study is that both CD40 and CD40L protein expression were higher in monocytes from patients with MS, compared with controls. The higher expression of the receptor CD40 in monocytes of patients with the MS indicates that these cells are more susceptible to be activated by CD40L, either in its soluble or cellular form. It is important to point out that the sCD40L is similar to the membrane-bound form in terms of its actions (26, 27). The interaction of the CD40L with its receptor participates in different inflammatory responses, such as the induction of proinflammatory cytokines, chemokines, matrix-degrading activities, and adhesion molecules on monocytes and endothelial cells (22, 28). Thus, up-regulation of CD40 may represent a mechanism of amplification of CD40L-induced proinflammatory pathways and the up-regulation of the cellular CD40L, an increased cell-cell interaction between atheroma-as-
associated cells. In other words, elevated expression of CD40 and CD40L at sites of chronic inflammation could further contribute to the inflammatory process of patients with the MS.

We next investigated the role of platelets as a source of sCD40L in the MS. CD40L rapidly translocates to the platelet surface after stimulation by thrombin, ADP, or collagen and then is enzymatically cleaved generating the soluble fragment (4, 26). 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**FIG. 5.** Regulation of CD40 and CD40L expression by AN on endothelial cells. Cells were incubated, for 24 h, in the presence or absence AN (30 μg/ml), and the levels of CD40 and CD40L were determined by real-time RT-PCR (A) or Western blotting (a representative Western blot is shown) (B). When indicated, an overnight pretreatment with TNF-α (50 ng/ml) was performed. The experiment was performed in triplicate. Bars represent mean value ± SEM. *, P < 0.05.

vascular cells (33) but, importantly, provide evidence that AN down-regulates the CD40/CD40L system, in both macrophages and endothelial cells. This study reports on what we believe is a novel antiinflammatory and antiatherogenic mechanism of the vascular protective action of AN. Because the molecular events involved in CD40L down-regulation by AN in monocytes and HUVECs remain to be fully elucidated, a detailed knowledge is essential for the development of novel treatment strategies in metabolic disorders.

**Limitations and perspectives of the study**

We used BMI to classify individuals in the current study because waist circumference measures were not available. Our study confirms enhanced sCD40L levels in the MS, although we failed to find any association between sCD40L levels and carotid IMT in these patients or with the presence of atheromatous plaques. There are contradictory data in the literature regarding the association of circulating sCD40L levels and the presence of subclinical atherosclerosis (38), but our results are in agreement with those obtained by de Lemos et al. (39) in a large representative sample.

Future studies will be required to further evaluate which mediators of the MS are involved in the activation of both the cel-
lar and soluble CD40L pathways. It has been suggested that early regulation of plasma sCD40L levels by treatment of obesity (40, 41) and diabetes (9) could result in lowering the risk of CV disease. Direct pharmacologic manipulation of the axis CD40/CD40L has also been tried with promising results in some cases (42, 43). Future studies will be needed to further clarify whether the ability of AN to directly modulate the CD40/CD40L system may represent an important mechanistic basis of CV disease in patients with the MS.

Summary

Our clinical and experimental study shows elevated circulating sCD40L levels, likely derived from activated platelets in patients with the MS. In addition to the soluble form, we show for the first time that the monocyte CD40/CD40L system is also up-regulated in patients with the MS, indicating that this dyad, by amplifying and sustaining inflammation, may contribute to CV risk in these patients. Also, we describe a new antiinflammatory effect of AN through the modulation of the CD40/CD40L system, in both macrophages and endothelial cells, that could be a novel mechanism through which AN may protect the vasculature.

Acknowledgments

We acknowledge Sonia Auseré for her skillful assistance in this project.

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This work was supported by the agreement between FIMA and “Unión Temporal de Empresas (UTE) project Center for Applied Medical Research,” by Grants 35/2005 from the Department of Health of the Government of Navarra, the Department of Education of the Government of Navarra, SAF2005-05919 from the Ministry of Science and Education, and Red Temática de Investigación Cooperativa en Enfermedades (RECAVA) RD06/0114/0008 from the Instituto de Salud Carlos III, Ministry of Health, Spain.

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