Original Article

The Ubiquitin-Proteasome System and Inflammatory Activity in Diabetic Atherosclerotic Plaques

Effects of Rosiglitazone Treatment

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The role of ubiquitin-proteasome system in the accelerated atherosclerotic progression of diabetic patients is unclear. We evaluated ubiquitin-proteasome activity in carotid plaques of asymptomatic diabetic and nondiabetic patients, as well as the effect of rosiglitazone, a peroxisome proliferator–activated receptor (PPAR)-γ activator, in diabetic plaques. Plaques were obtained from 46 type 2 diabetic and 30 nondiabetic patients undergoing carotid endarterectomy. Diabetic patients received 8 mg rosiglitazone (n = 23) or placebo (n = 23) for 4 months before scheduled endarterectomy. Plaques were analyzed for macrophages (CD68), T-cells (CD3), inflammatory cells (HLA-DR), ubiquitin, proteasome 20S activity, nuclear factor (NF)-κB, inhibitor of κB (IκB)-β, tumor necrosis factor (TNF)-α, nitrotyrosine, matrix metalloproteinase (MMP)-9, and collagen content (immunohistochemistry and enzyme-linked immunosorbent assay). Compared with nondiabetic plaques, diabetic plaques had more macrophages, T-cells, and HLA-DR+ cells (P < 0.001); more ubiquitin, proteasome 20S activity (TNF-α), and NF-κB (P < 0.001); and more markers of oxidative stress (nitrotyrosine and O_2^- production) and MMP-9 (P < 0.01), along with a lesser collagen content and IκB-β levels (P < 0.001). Compared with placebo-treated plaques, rosiglitazone-treated diabetic plaques presented less inflammatory cells (P < 0.01); less ubiquitin, proteasome 20S, TNF-α, and NF-κB (P < 0.01); less nitrotyrosine and superoxide anion production (P < 0.01); and greater collagen content (P < 0.01), indicating a more stable plaque phenotype. Similar findings were obtained in circulating monocytes obtained from the two groups of diabetic patients and cultured in the presence or absence of rosiglitazone (7.0 μmol/l). Ubiquitin-proteasome over-activity is associated with enhanced inflammatory reaction and NF-κB expression in diabetic plaques. The inhibition of ubiquitin-proteasome activity in atherosclerotic lesions of diabetic patients by rosiglitazone is associated with morphological and compositional characteristics of a potential stable plaque phenotype, possibly by downregulating NF-κB-mediated inflammatory pathways. *Diabetes* 55:622–632, 2006

Cardiovascular disease represents the leading cause of death in patients with type 2 diabetes (1). Diabetes leads to increased vulnerability for plaque disruption and mediates increased incidence and severity of clinical events (2). Inflammation, particularly in diabetes, plays a central role in the cascade of events that result in plaque erosion and fissuring (2). There is emerging evidence that the ubiquitin-proteasome system, the major pathway for nonlysosomal intracellular protein degradation in eucaryotic cells, induces inflammation in both initial stage and progression of atherosclerosis (3). The ubiquitin-mediated proteolytic pathway involves the conjugation of multiple moieties of ubiquitin, a 76-amino acid polypeptide, to cellular proteins in a multienzymatic process, targeting these proteins to degradation (4). This ligation of ubiquitin by a series of ubiquitin-conjugating enzymes produce polyubiquitin chains, which serve as targeting signals for degradation of the protein by the proteasome. The multicatalytic proteasome consists of a central catalytic core, the 20S proteasome, and two regulatory 19S complexes (5). Moreover, the ubiquitin-proteasome pathway is required for activation of nuclear factor kappa B (NF-κB), a central transcription factor that regulates inflammatory genes, by degradation of its inhibitory IκB proteins (6). Although it has been demonstrated that diabetes may upregulate ubiquitin-proteasome pathway in rat muscle (7), still no evidence exists about the potential role of ubiquitin-proteasome system in the evolution of atherosclerotic plaques of diabetic patients. We hypothesized that by increasing ubiquitin-proteasome activity, diabetes may enhance the inflammatory potential of atherosclerotic plaques favoring instability.

This study was designed to identify differences in in-

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Received for publication 30 June 2005 and accepted in revised form 8 December 2005.

CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; HOMA-IR, homeostasis model assessment of insulin resistance; IκB, inhibitor of κB; MMP, matrix metalloproteinase; NF, nuclear factor; PPAR, peroxisome proliferator–activated receptor; TNF, tumor necrosis factor.

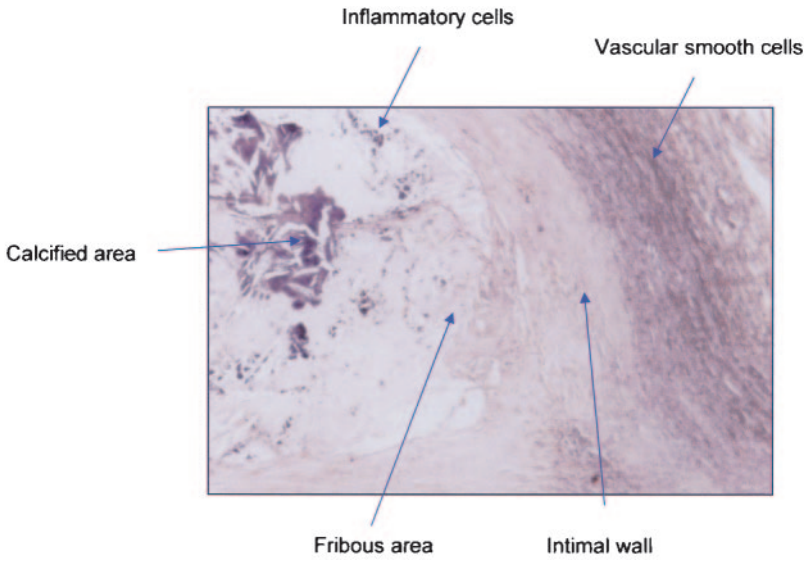

FIG. 1. Representative example of plaques studied. This section is representative of the plaque region analyzed. The section was stained with hematoxylin-eosin. Plaque section at low magnification.

flammatory infiltration as well as ubiquitin-proteasome activity between carotid plaques of asymptomatic diabetic and nondiabetic patients. Because experimental and pathological studies suggest that activation of peroxisome proliferator–activated receptor (PPAR)-γ may reduce inflammation (8) and inhibit ubiquitin-proteasome activity (9), the study also evaluated the effect of the PPAR-γ agonist rosiglitazone on ubiquitin-proteasome activity in carotid plaques of diabetic patients.

RESEARCH DESIGN AND METHODS

The study group comprised 46 type 2 diabetic and 30 nondiabetic patients (nondiabetic group) with asymptomatic carotid stenosis, enlisted to undergo carotid endarterectomy for extracranial high-grade (>70%) internal carotid artery stenosis (10). Diabetes was categorized according to American Diabetes Association criteria (11). The diabetic patients were randomized in a double-blind fashion to receive rosiglitazone (4 mg b.i.d., $n = 23$) or placebo ($n = 23$) for 4 months, according to human studies evaluating the carotid arterial intima-media thickness progression as well as circulating markers of inflammation after PPAR-γ agonist treatments (12,8). In the placebo group, the dose of other concomitant hypoglycemic medication was changed to obtain a similar profile of metabolic parameters. Additional antidiabetic therapy, including sulfonylurea, metformin, and insulin, was titrated for optimal glycemic control for 4 months. After the treatment period, all patients had undergone endarterectomy. Fasting plasma glucose, insulin, and serum lipids, as well as insulin resistance index (by homeostasis model assessment of insulin resistance [HOMA-IR]) (13), were measured at baseline, monthly, and before endarterectomy. Moreover, C-reactive protein (CRP) serum levels (by enzyme-linked immunosorbent assay [ELISA]; Diagnostic Systems Laboratory) were measured at baseline and before endarterectomy in 12 patients from the control group, 15 from the placebo group, and 16 from the rosiglitazone group. Written informed consent was obtained from all patients before each examination. The local ethics review committee approved the study.

Atherectomy specimens. After surgery, the specimens were cut perpendicular to the long axis into two halves. The first half was frozen in liquid nitrogen for the following ELISA analysis. A portion of the other half specimen was immediately immersion-fixed in 10% buffered formalin. Sections were serially cut at 5 μm, mounted on lysine-coated slides, and stained with hematoxylin and eosin and with the trichrome method. Carotid artery specimens were analyzed by light microscopy.

Immunohistochemistry. After the surgical procedure, samples were immediately frozen in isopentane and cooled in liquid nitrogen. Similar regions of the plaque were analyzed (Fig. 1). Serial sections were incubated with specific antibodies: anti-ubiquitin, anti–proteasome 20S, anti–HLA-DR, anti-CD68, and anti-CD3 (Dako); anti–IκB-β and anti-matrix metalloproteinase (MMP)-9 (Santa Cruz); and anti–tumor necrosis factor (TNF)-α (R&D). Specific antibodies that selectively recognize the activated form of NF-κB (p65 and p50 subunits; Santa Cruz) were used. Analysis of immunohistochemistry was performed with a personal computer-based quantitative 24-bit color image analysis system (IM500; Leica Microsystem AG).

Biochemical assays. Plaques were lysed and centrifuged for 10 min at 10,000*g* at 4°C. After centrifugation, 20 μg of each sample were loaded, electrophoresed in polyacrylamide gel, and electroblotted onto a nitrocellulose membrane. Each determination was repeated at least three times. Ubiquitin, IκB-β, MMP-9, TNF-α, and nitrotyrosine levels were quantified in plaques using a specific ELISA kits (from Santa Cruz, R&D Systems, and Imgenex). Nuclear extracts from plaque specimens were obtained as described by Ohlsson et al. (14). We used a specific antibody that selectively recognizes the activated form of the NF-κB subunit p65. NF-κB binding to κB sites was assessed using the Trans-AM NF-κB p65 transcription factor assay kit (Active Motif Europe; Rixensart). In this assay, an oligonucleotide containing the NF-κB consensus site is attached to a 96-well plate. The active form of NF-κB contained in cell extracts specifically binds to this oligonucleotide and can be revealed by incubation with antibodies. Thereafter, whole-cell extracts were prepared, and 10 μg of total cellular proteins were analyzed for p65 binding to κB oligonucleotide according to the manufacturer's instructions. In addition, we analyzed the expression of the activated p50 subunit by specific Trans-AM NF-κB p50 transcription factor assay kit (Active Motif; Rixensart). For the quantitative measurement of the proteasome 20S activity, a specific SDS activation kit (Boston Biochem) was used. Nitrotyrosine was assayed into the plaque tissue with a kit supplied by Hycult Biotech.

Macrophages extraction from atherosclerotic plaques. Macrophages were selectively extracted from plaques as described by de Vries et al. (15). Biochemical assay on cell homogenates for ubiquitin and proteasome 20S determinations were performed as illustrated earlier.

Measurement of O_2^-. Production of O_2^- was measured as the superoxide dismutase–inhibitable reduction of cytochrome *c*, as previously described (16)

Isolation and culture of blood monocytes. Peripheral blood monocytes from 10 diabetic and 10 nondiabetic patients were purified and cultured as described by Cipollone et al. (17). Monocytes from diabetic patients (24×10^6/4 ml of Dulbecco's modified Eagle's medium) were cultured in the presence or absence of rosiglitazone (7.0 μmol/l for 48 h) and in the presence or absence of MG132 (10 mmol/l stock solutions in DMSO; Calbiochem), a specific proteasome inhibitor (10 μmol/l for 5 h). At the end of the incubation, adherent monocytes were scraped, collected, and lysed, and ubiquitin, proteasome 20S, NF-κB, IκB-β, and O_2^- production were evaluated.

Statistical analysis. Data are presented as mean ± SD. Continuous variables were compared among the groups of patients with one-way ANOVA for normally distributed data and Kruskal-Wallis for non–normally distributed data. When differences were found among the groups, Bonferroni correction was used to make pairwise comparisons. $P \leq 0.05$ was considered statistically significant. All calculations were performed using SPSS 12.

RESULTS

Demographic data for the study population are presented in Table 1. Percentage of carotid diameter reduction, risk factors, and concomitant nonhypoglycemic therapy did not differ among the groups (Table 1). In diabetic patients, mean plasma glucose between weeks 0 and 16 decreased similarly with placebo (−1.6 ± 0.6 mmol/l, $P < 0.05$) and rosiglitazone (−1.5 ± 0.6 mmol/l, $P < 0.05$), while HbA_{1c}

TABLE 1
Characteristics of study patients

Variables	Control group	Placebo group	Rosiglitazone group
n	30	23	23
Age (years)	69 ± 2	68 ± 3	69 ± 2
Male	16 (53.3)	13 (56.5)	12 (52.2)
Patients characteristics			
Family history of IHD	14 (46.7)	13 (56.5)	16 (69.6)
Hypertension	15 (50)	11 (47.8)	10 (43.5)
Hypercholesterolemia	13 (43.3)	10 (43.5)	9 (39.1)
Cigarette smoking	12 (40)	7 (30.4)	8 (34.8)
Coronary artery disease	13 (43.3)	14 (60.9)	16 (69.6)
BMI (kg/m^2)	27.6 ± 2	28.2 ± 2	28.9 ± 3
Systolic blood pressure (mmHg)	128 ± 12	129 ± 11	130 ± 12
Diastolic blood pressure (mmHg)	81 ± 4	81 ± 4	82 ± 3
A1C (%)	4.9 ± 1.1*†	8.1 ± 1.4	8.2 ± 1.5
Blood glucose (mmol/l)	6 ± 0.9*†	9.7 ± 1.4	9.6 ± 1.3
Insulin (μU/ml)	8.06 ± 3.3*†	10.6 ± 2.4	10.8 ± 2.7
HOMA-IR	2.15 ± 0.9*†	4.57 ± 1.3	4.61 ± 1.3
CRP (mg/dl)	0.86 ± 0.09*†	1.22 ± 0.08	1.19 ± 0.09
Total cholesterol (mmol/l)	5.64 ± 0.09	5.63 ± 0.09	5.67 ± 0.10
HDL cholesterol (mmol/l)	1.26 ± 0.10	1.22 ± 0.08	1.21 ± 0.09
Triglycerides (mmol/l)	1.94 ± 0.33	1.95 ± 0.31	1.97 ± 0.34
Stenosis severity (%)	75.5 ± 4.9	76.4 ± 4.5	76.2 ± 3.8
Macrophage-rich areas (%)	5 ± 2*†	24 ± 5	14 ± 6*
T-cells per mm^2 section area	15 ± 7*†	70 ± 13	41 ± 12*
HLA-DR (%)	12 ± 3*†	31 ± 5	19 ± 6*
Active therapy			
Aspirin	24 (80)	21 (91.3)	20 (86.9)
Warfarin	2 (6.6)	1 (4.3)	1 (4.3)
β-Blocker	7 (23.3)	5 (21.7)	5 (21.7)
Calcium-channel blocker	3 (10)	4 (17.4)	3 (13.1)
Statin	20 (66.6)	17 (73.9)	16 (69.6)
ACE inhibitor	16 (53.3)	14 (60.9)	12 (52.1)
Diuretic agent	9 (30)	6 (26.1)	7 (30.4)
AT-2 antagonist	14 (46.6)	12 (52.1)	11 (48.7)
Insulin	—	16 (69.6)	17 (73.9)
Sulfonylureas	—	8 (34.8)	7 (30.4)
Metformin	—	6 (26.1)	5 (21.7)

Data are means ± SD or *n* (%). *$P < 0.05$ vs. the placebo group; †$P < 0.05$ vs. the rosiglitazone group. IHD, ischemic heart disease.

(A1C) levels showed a trend to decrease in both groups (−0.83 ± 0.4%, $P = 0.14$, and −0.87 ± 0.5%, $P = 0.16$, respectively). In the placebo group, glimepiride was tested and its daily dose was adjusted in four patients to a range of 1–6 mg/day. Metformin was adjusted during the study in three patients; the daily dose range was 500–1,700 mg/day. Insulin was adjusted in nine patients. HOMA-IR decreased in the rosiglitazone group but remained unchanged in the placebo group (−1.1 ± 0.3 vs. −0.4 ± 0.2, $P < 0.01$). In diabetic patients, the decline in CRP was −0.12 mg/dl in the placebo group and −0.42 mg/dl in the rosiglitazone group (Table 2). No patient in either group developed any clinical events during the study.

Plaque composition. Compared with nondiabetic patients, diabetic patients (all) had a significantly greater portion of plaque area occupied by macrophages (19 ± 5 vs. 5 ± 2%; $P < 0.01$) and T-cells (55 ± 13 vs. 15 ± 7%; $P < 0.01$), as well as greater expression of HLA-DR antigen (25 ± 5 vs.12 ± 3%, $P < 0.01$) (Table 1 and Fig. 2). Compared with the placebo group, the rosiglitazone group presented a significantly smaller portion of plaque area occupied by macrophages ($P < 0.01$) and T-cells ($P < 0.01$), as well as lower expression of HLA-DR ($P < 0.01$) (Table 1 and Fig. 2). Both immunohistochemistry and ELISA revealed markedly higher staining and levels of TNF-α in all diabetic lesions compared with nondiabetic lesions ($P < 0.001$). In diabetic patients, staining and levels of TNF-α were significantly more abundant in lesions from placebo than lesions from the rosiglitazone group ($P < 0.001$) (Fig. 2). MMP-9 levels were more abundant in diabetic than in nondiabetic lesions ($P < 0.001$); in diabetic patients, MMP-9 levels were more abundant in lesions from the placebo group than those from the rosiglitazone group ($P < 0.001$). Lower content of interstitial collagen was found in plaques of all diabetic patients compared with nondiabetic patients. Content of interstitial collagen of plaques from rosiglitazone-treated patients was higher compared with that from placebo-treated patients ($P < 0.01$) (Fig. 2).

Ubiquitin-proteasome activity. Immunohistochemistry revealed higher staining of ubiquitin and proteasome 20S in diabetic inflammatory cells than in nondiabetic inflammatory cells ($P < 0.01$), with the highest staining detected in patients randomized to placebo. A similar pattern of response was seen for ubiquitin plaque levels ($P < 0.001$) and proteasome 20S plaque activity ($P < 0.001$) (Fig. 3).

Ubiquitin and proteasome 20S in macrophages extracted from plaques. To identify whether the higher ubiquitin-proteasome levels observed in diabetic plaques were produced by macrophages, we repeated quantitative

TABLE 2
Metabolic parameters of diabetic patients after rosiglitazone and placebo treatments

Variables	Placebo group	Rosiglitazone group
n	23	23
BMI (kg/m^2)	27.9 ± 7	29.1 ± 5
Systolic blood pressure (mmHg)	126 ± 13	126 ± 15
Diastolic blood pressure (mmHg)	82 ± 6	80 ± 4
A1C (%)	7.3 ± 1.5	7.3 ± 1.8
Blood glucose (mmol/l)	8.1 ± 1.7*	8.1 ± 1.9*
Insulin (μU/ml)	10.6 ± 2.7	10.4 ± 2.9
HOMA-IR	4.47 ± 1.5	3.51 ± 1.2*†
CRP (mg/dl)	1.1 ± 0.07	0.77 ± 0.09*†
Total cholesterol (mmol/l)	5.61 ± 0.11	5.69 ± 0.14
HDL cholesterol (mmol/l)	1.24 ± 0.09	1.29 ± 0.1
Triglycerides (mmol/l)	1.85 ± 0.32	1.89 ± 0.36

Data are means ± SD. *$P < 0.05$ vs. baseline data; †$P < 0.05$ vs. the placebo group.

analyses on macrophages selectively extracted from seven plaques randomly selected from each of the three groups. The small sample size used was not sufficient to reach an adequate statistical power for the comparisons among groups, so we can only describe the tendency of central measures for the three groups. However, we observed that the placebo group had the highest of both ubiquitin levels (457.9 ± 99 ng/mg) and proteasome 20S activity (76.3 ± 22 pmol/mg); the nondiabetic group had the lowest levels (ubiquitin 237 ± 78 ng/mg, proteasome 20S 27.6 ± 6 pmol/mg), and rosiglitazone had intermediate levels (ubiquitin 339 ± 89 ng/mg, proteasome 20S 41.9 ± 12 pmol/mg).

Colocalization of proteasome 20S and ubiquitin with macrophages in diabetic plaques. Serial sections of diabetic plaques were incubated with the primary antibodies anti-ubiquitin, anti–proteasome 20S, and anti-CD68. The expression of both ubiquitin and proteasome 20S were associated with CD68+ macrophages in plaque sections (Fig. 4). Thus, these analyses confirmed the concomitant presence of ubiquitin and proteasome 20S in macrophages of diabetic plaque.

NF-κB activity. NF-κB activation, as reflected by the selective analysis of the activated form of both p50 and p65, was significantly higher in both placebo ($P < 0.01$) and rosiglitazone ($P < 0.01$) inflammatory cells than in nondiabetic macrophages. In plaques from diabetic patients, both p50 ($P < 0.01$) and p65 ($P < 0.001$) were significantly higher in placebo-treated than in rosiglitazone-treated patients. Immunohistochemistry and quantitative analyses revealed lower staining and levels for IκB-β in diabetic inflammatory cells than in nondiabetic inflammatory cells (placebo $P < 0.01$, rosiglitazone $P < 0.001$). Moreover, both staining for and levels of IκB-β were higher in rosiglitazone than in placebo plaques (Fig. 5).

Quantification of oxidative stress. Higher nitrotyrosine levels were found in diabetic plaques than in nondiabetic plaques (diabetic placebo 3.4 ± 0.43 nmol/pg, diabetic rosiglitazone 2.4 ± 0.23 nmol/pg, nondiabetic group 1.2 ± 0.31 nmol/pg, $P < 0.001$). A similar pattern was found for O_2^- production (diabetic placebo 6.22 ± 1.5 pmol/l, diabetic rosiglitazone 3.87 ± 1.2 pmol/l, nondiabetic group 2.34 ± 0.98 pmol/l, $P < 0.01$). In diabetic patients, nitrotyrosine levels and O_2^- production were significantly higher in plaques from placebo-treated than in rosiglitazone-treated patients (nitrotyrosine 3.4 ± 0.43 vs. 2.4 ± 0.23 nmol/pg, $P < 0.01$; O_2^- 6.22 ± 1.5 vs. 3.87 ± 1.2 pmol/l, $P < 0.01$).

In vitro study. Monocytes from diabetic patients evidenced higher levels of ubiquitin, p50, p65, and O_2^- production, as well as higher proteasome 20S, along with a lesser IκB-β levels compared with monocytes from nondiabetic patients ($P < 0.01$). Levels of ubiquitin, p50, p65, and O_2^- production, as well as proteasome 20S activity, were significantly lower in the diabetic monocytes incubated with rosiglitazone than in diabetic monocytes incubated without rosiglitazone ($P < 0.01$), whereas IκB-β levels were significantly higher in the diabetic monocytes incubated with rosiglitazone than in diabetic monocytes incubated without rosiglitazone ($P < 0.01$) (Fig. 6). Levels of p50 and p65 levels were significantly lower in the diabetic monocytes incubated with MG132 than in those incubated without MG132 (p50 16.8 ± 1.3 vs. 20 ± 1.6 ng/mg, $P < 0.01$; p65 25 ± 3.2 vs. 30.4 ± 1.5 ng/mg, $P < 0.01$), whereas IκB-β (32.8 ± 8.7 vs. 19 ± 2.4 ng/mg, $P < 0.01$) was significantly higher in the diabetic monocytes incubated with MG132 than in those incubated without MG132. Although ubiquitin levels were higher in monocytes incubated with MG132, there were not statistically significant differences among the groups (434.7 ± 38.5 vs. 410 ± 44.4 ng/mg, $P = 0.2$).

DISCUSSION

This study shows that ubiquitin-proteasome system overactivity is associated with the inflammatory process of atherosclerotic plaques of type 2 diabetic patients. In particular, the ubiquitin-proteasome activity was greater in diabetic atherosclerotic lesions as compared with nondiabetic lesions, and it was associated with higher NF-κB and MMP-9 levels along with less interstitial collagen content. All this might increase the risk of future acute ischemic events precipitated by inflammatory-dependent rupture of atherosclerotic plaques. Moreover, we provide evidence that the PPAR-γ agonist rosiglitazone may prevent plaque progression to an unstable phenotype in diabetic patients by reducing ubiquitin-proteasome activity.

A previous postmortem study (18) has reported enhanced ubiquitin expression in unstable coronary plaques. However, the study did not provide any evidence about ubiquitin expression in subgroups of high-risk plaques, such as those found in diabetic patients, or assess the specific pathway transducing environmental stimuli in ubiquitin-proteasome overexpression. In our study, macrophages, T-cells, and HDLA-DR+ inflammatory cells were more abundant in diabetic plaques and represented the major source of ubiquitin-proteasome activity, suggesting the presence of an active inflammatory reaction in diabetic plaques. Concomitantly higher expression of ubiquitin and proteasome was found in human plaque macrophages obtained from the asymptomatic carotid lesions of patients with type 2 diabetes compared with nondiabetic lesions. In agreement with the difference in ubiquitin-proteasome staining pattern, the histological milieu of the lesions appears different with regard to cellularity, but not in the degree of vessel stenosis, suggesting that diabetic and nondiabetic lesions are only different in regard to inflammatory burden. These data are consistent with previous findings that the inflammatory response was greater in diabetic

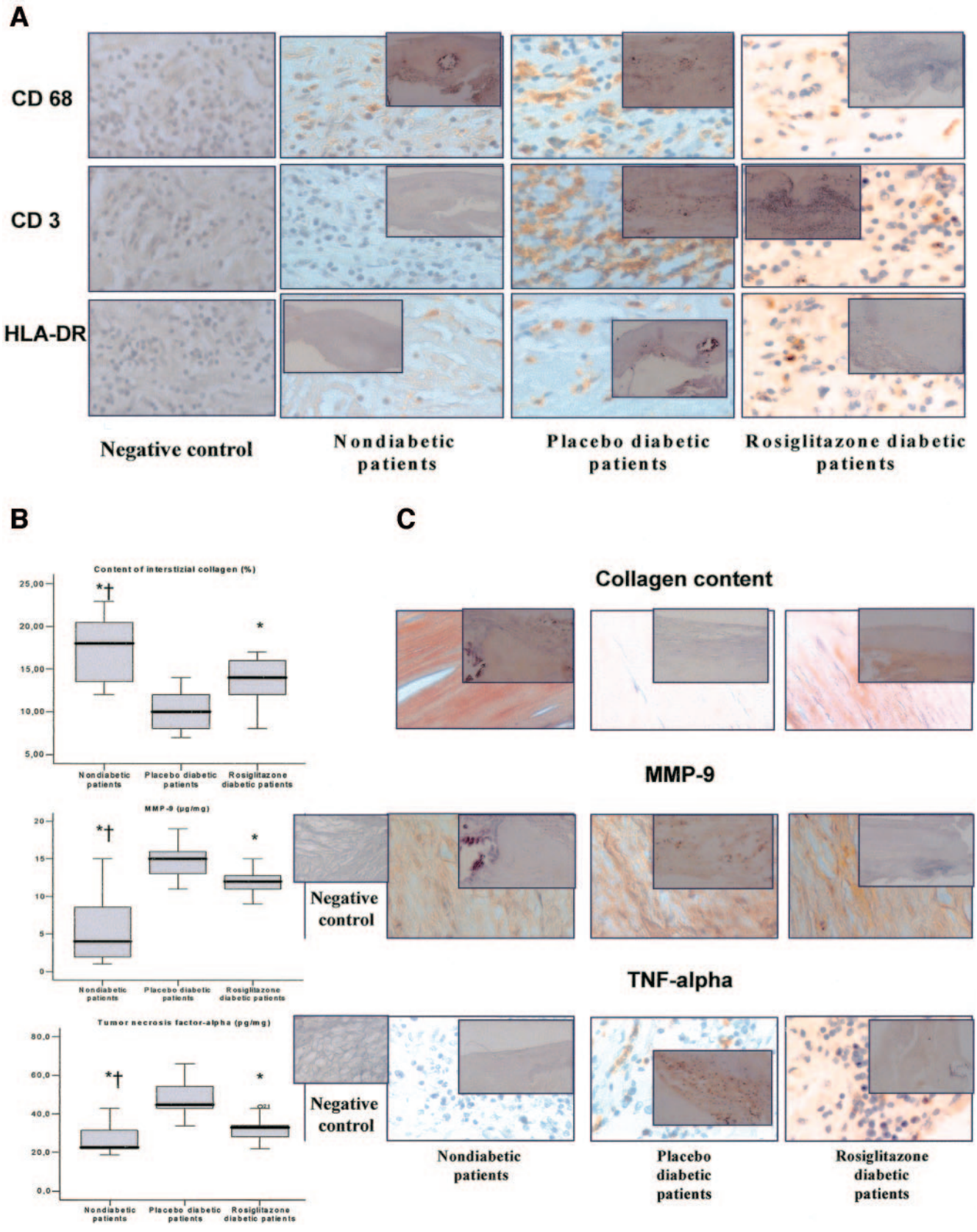

FIG. 2. ***A*****: Representative sections show immunochemistry for CD68, CD3, and HLA-DR in nondiabetic, placebo-treated, and rosiglitazone-treated diabetic plaques. The boxes show the immunochemistry at low magnification.** ***B*****: ELISA for MMP-9, TNF-α, and sirius red staining for collagen content in nondiabetic, placebo-treated, and rosiglitazone-treated diabetic plaques. (The boxplots show the median, 25th, and 75th percentiles, range, and extreme values.)** ***C*****: Representative sections show immunochemistry for MMP-9, TNF-α, and sirius red staining for collagen content in nondiabetic, placebo-treated, and rosiglitazone-treated diabetic plaques. The boxes show the immunochemistry at low magnification. Similar regions of plaque are shown. These results are typical of nondiabetic, placebo-treated, and rosiglitazone-treated diabetic plaques. *$P < 0.05$ vs. the placebo group; †$P < 0.05$ vs. the rosiglitazone group.**

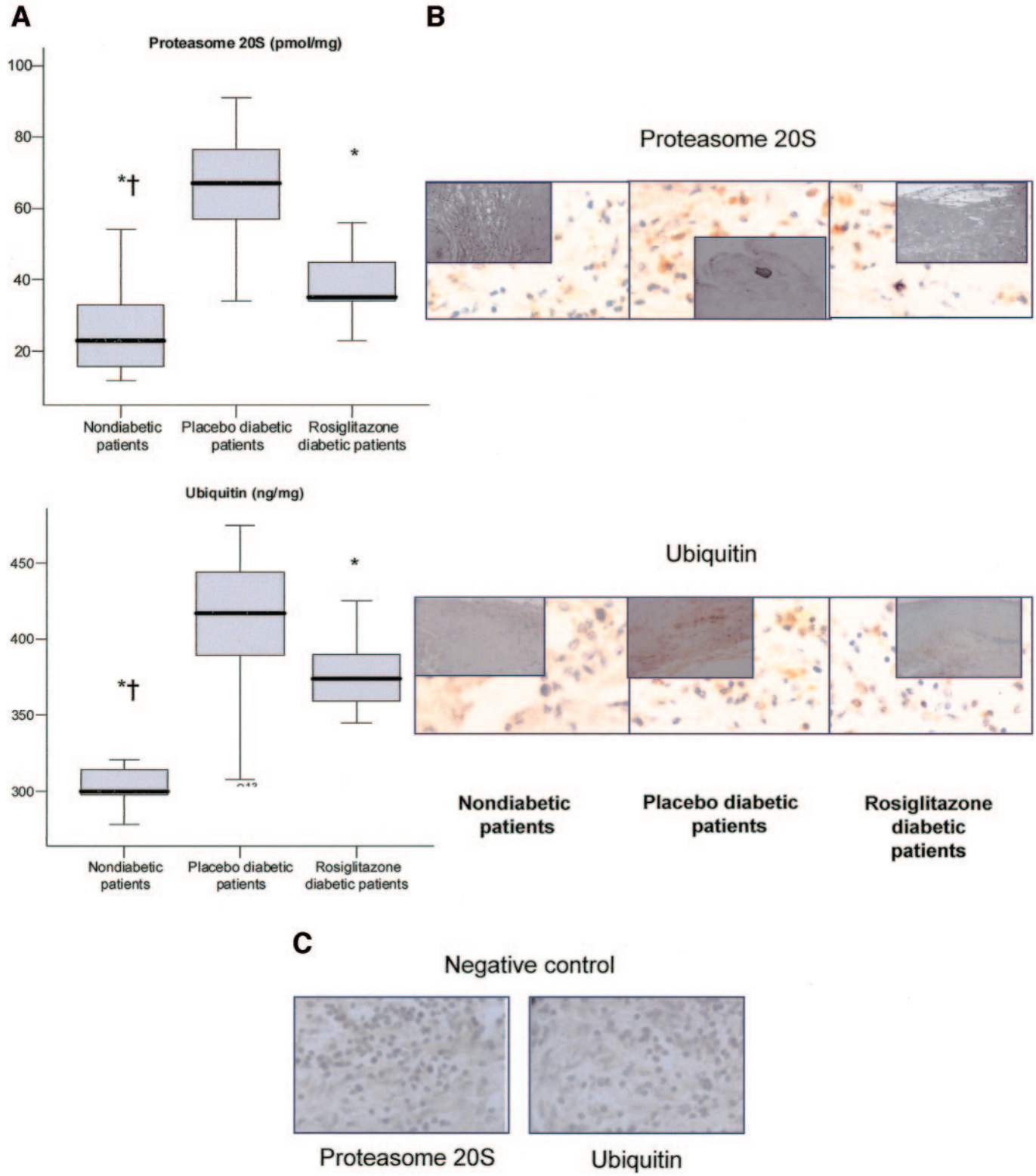

FIG. 3. ***A*****: Levels of proteasome 20S by a specific SDS activation kit and ubiquitin levels by an ELISA kit.** ***B*****: Proteasome 20S and ubiquitin by immunohistochemistry in nondiabetic, placebo-treated, and rosiglitazone-treated diabetic plaques. The boxes show the immunochemistry at low magnification.** ***C*****: Negative control. Similar regions of the plaque are shown. These results are typical of nondiabetic, placebo-treated, and rosiglitazone-treated diabetic plaques. *$P < 0.05$ vs. the placebo group; †$P < 0.05$ vs. the rosiglitazone group.**

than in nondiabetic plaques (17). Moreover, an association between RAGE-expressing macrophages and upregulation of inflammatory NF-κB, COX-2/mPGES-1, and MMPs has been shown in carotid arteries of diabetic patients (17). Finally, a recent postmortem study (19) demonstrates an increase in apoptotic macrophages and smooth muscle cells in coronary arteries of diabetic patients, which may be related to necrotic core expansion, thinning of the fibrous cap, and plaque instability. Hence, the differences in plaque behavior likely stem

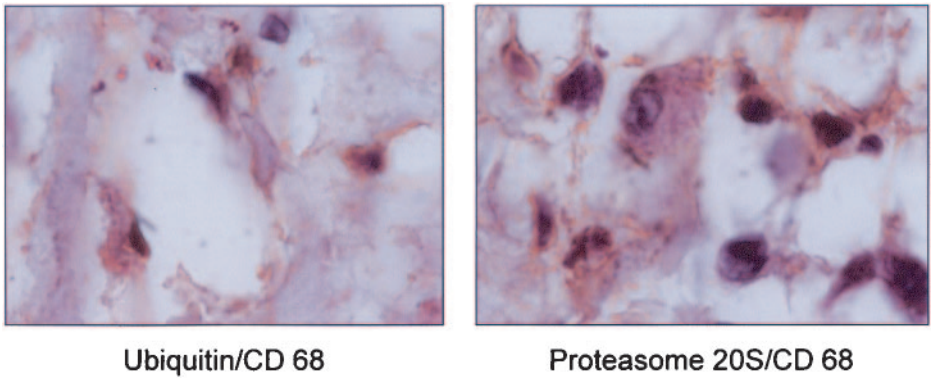

FIG. 4. Diabetic plaque sections show that ubiquitin and proteasome 20S colocalize with macrophage immunoreactivity.

from differences in the presence of stimuli (i.e., oxidative stress, as evidenced by high O_2^- production and nitrotyrosine levels) for selective expression of ubiquitin-proteasome, capable of disrupting plaque stability via NF-κB induction.

NF-κB is normally bound to IκB in the cytosol; this binding prevents its movement into the nucleus (20). Various cellular stimuli, such as oxidative stress, induce ubiquitination of phosphorylated IκBs and subsequent degradation by the proteasome (21). Degradation of IκBs results in unmasking of the nuclear localization signal of NF-κB dimers, which subsequently translocates to the nucleus, where it induces the transcription of proinflammatory cytokines that play a central role in plaque instability progression, like TNF-α (22,23). Our findings also suggest that diabetes may induce phosphorylation and degradation of IκBs via the ubiquitin-proteasome overactivity, thus enhancing NF-κB activation. Previous reports evidenced the involvement of the ubiquitin-proteasome system in NF-κB activation, particularly under conditions of aggravated oxidative stress. Oxidative stress is the common factor underlying insulin resistance, type 2 diabetes, and cardiovascular disease and may explain the presence of inflammation in each of these conditions (24). Although it is well recognized that inflammation is one manifestation of oxidative stress, and the pathways that generate the mediators of inflammation, such as interleukins, are all induced by oxidative stress (25), the mechanism by which oxidative stress may be involved in inflammatory process of type 2 diabetic plaques is not fully clarified. In this context, our data suggest a novel mechanism by which oxidative stress, increasing ubiquitin-proteasome activity, may mediate inflammatory activity in diabetic atherosclerotic plaques. Of note, it has been shown that oxidative stress can stimulate the ubiquitin system in macrophages by inducing the expression of components of its enzymatic machinery, such as ubiquitin-binding proteins (26,27). Accordingly, in cultured monocytes from diabetic patients, we evidenced that O_2^- production, as well as ubiquitin-proteasome activity and NF-κB levels, were significantly higher when compared with nondiabetic patients. Thus, we can speculate that increased ubiquitin-proteasome activity in plaque macrophages, as a consequence of oxidative stress overexpression, may enhance the synthesis of NF-κB in the same cell, possibly representing a crucial step in the pathophysiology of diabetic plaque instability. Because of the study design, we cannot exclude whether the ubiquitin-proteasome pathway exerts also a protective compensatory response (28), or whether it is merely a correlative marker for the presence of inflammatory cells in diabetic lesions. However, the concomitant presence of ubiquitin and proteasome 20S in diabetic macrophages, as well as the reduction of NF-κB levels in diabetic monocytes treated with proteasome inhibitor, suggests that the ubiquitin-proteasome pathway may have a proinflammatory effect in diabetic individuals. Moreover, higher expression of ubiquitin-proteasome and MMP-9 in diabetic lesions, one of the most important enzymes in the process of atherosclerotic plaque rupture (29), along with a lesser interstitial collagen content, suggests an involvement of the ubiquitin proteasome system in instability of diabetic lesion by increasing plaque erosion.

The present findings also show an inhibitory effect of rosiglitazone on ubiquitin-proteasome activity in diabetic lesions. Indeed, at the same level of blood glucose levels, diabetic patients treated with rosiglitazone had the lowest level of ubiquitin and proteasome 20S activity, plaque inflammatory cells, cytokines, oxidative stress, and MMP-9 associated with the highest content of plaque interstitial collagen. Thus, patients assigned to rosiglitazone had lesser plaque progression to an unstable phenotype than patients assigned to placebo. In particular, the reduced ubiquitin-proteasome activity seen in diabetic plaques of the rosiglitazone group suggests decreased IκB degradation and hence NF-κB activation. All this may have clinical implications because in a large series of carotid endarterectomy specimens, it has been shown that plaque inflammation is one of the major determinants of ischemic events in patients affected by carotid atherosclerotic disease (30). Sufficient data in humans suggest that PPAR-γ agonists reduce common carotid arterial intima media thickness progression as well as circulating markers of inflammation as early as 3 months after the administration in both nondiabetic and diabetic patients (8,12). Moreover, there is accumulating data to suggest that PPAR-γ agonists exert anti-inflammatory and antioxidant effects, including decreases of cytokines and MMPs in monocytes, macrophages, T-cells, and vascular smooth muscle cells (31–35). In line with this evidence, we observed a significant reduction of CRP serum levels in diabetic patients treated with rosiglitazone. Although PPAR-γ agonists may inhibit proteasome activity in human cancer (36), until now there was no evidence that this effect was reproducible in human atherosclerotic plaques. The hypothesis that plaque ubiquitin-proteasome activity is reduced by PPAR-γ agonists is also supported by our "in vitro experiments" that evidenced a reduction of ubiquitin-proteasome activity, NF-κB levels, and O_2^- production associated with a significant increment of IκB-β, in monocytes from diabetic patients treated with rosiglitazone. The mechanism of repression of the proteasome by rosiglitazone remains a

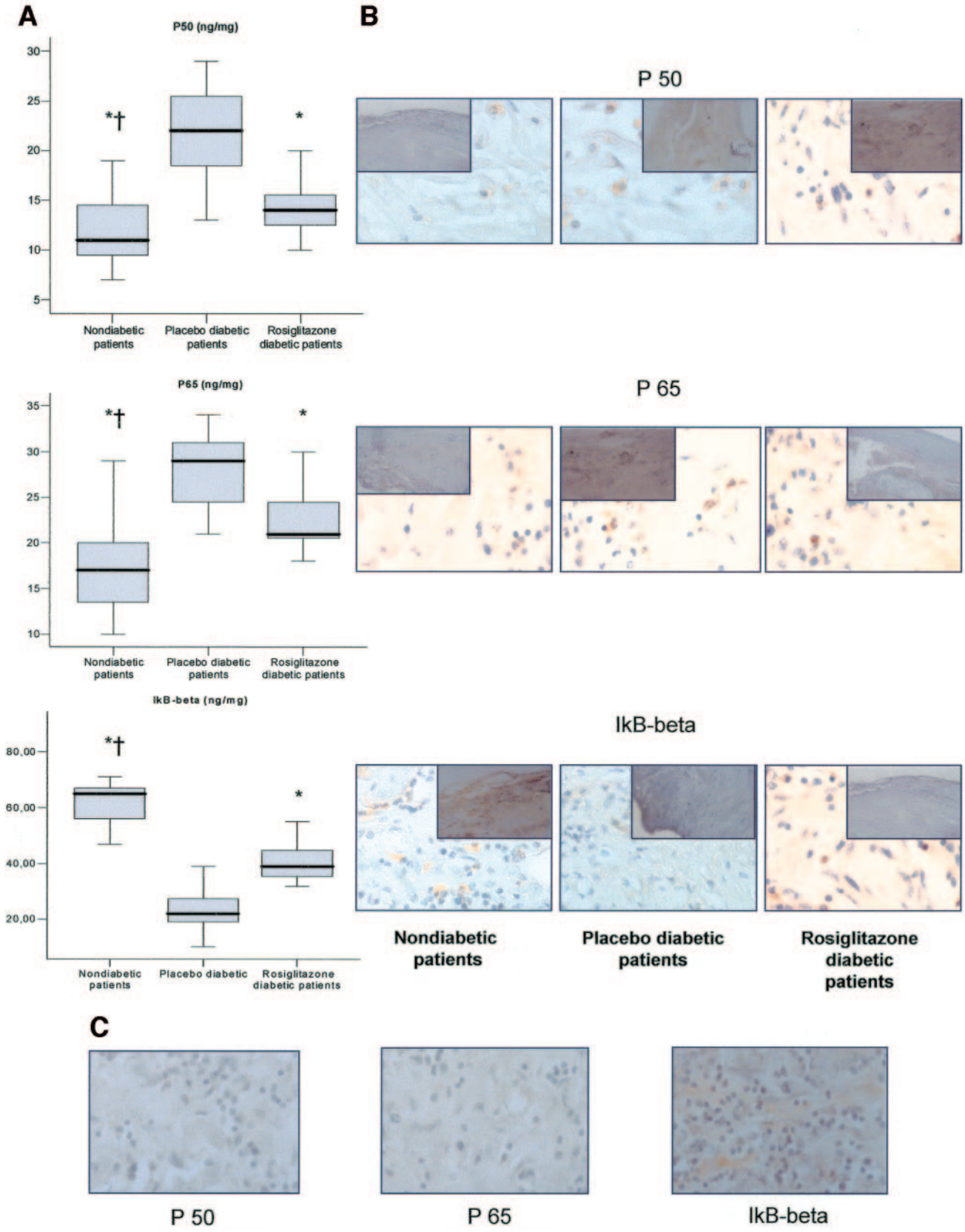

FIG. 5. ***A*****: Levels of activated NF-κB (specific Trans-AM p50 and p65 subunit assay kit) and IκB-β in nondiabetic, placebo-treated, and rosiglitazone-treated diabetic plaques.** ***B*****: Representative sections show immunohistochemistry for activated p65 and p50 and IκB-β. The boxes show the immunochemistry at low magnification.** ***C*****: Negative control. Similar regions of the plaque are shown. These results are typical of nondiabetic, placebo-treated, and rosiglitazone-treated diabetic plaques. *$P < 0.05$ vs. the placebo group; †$P < 0.05$ vs. the rosiglitazone group.**

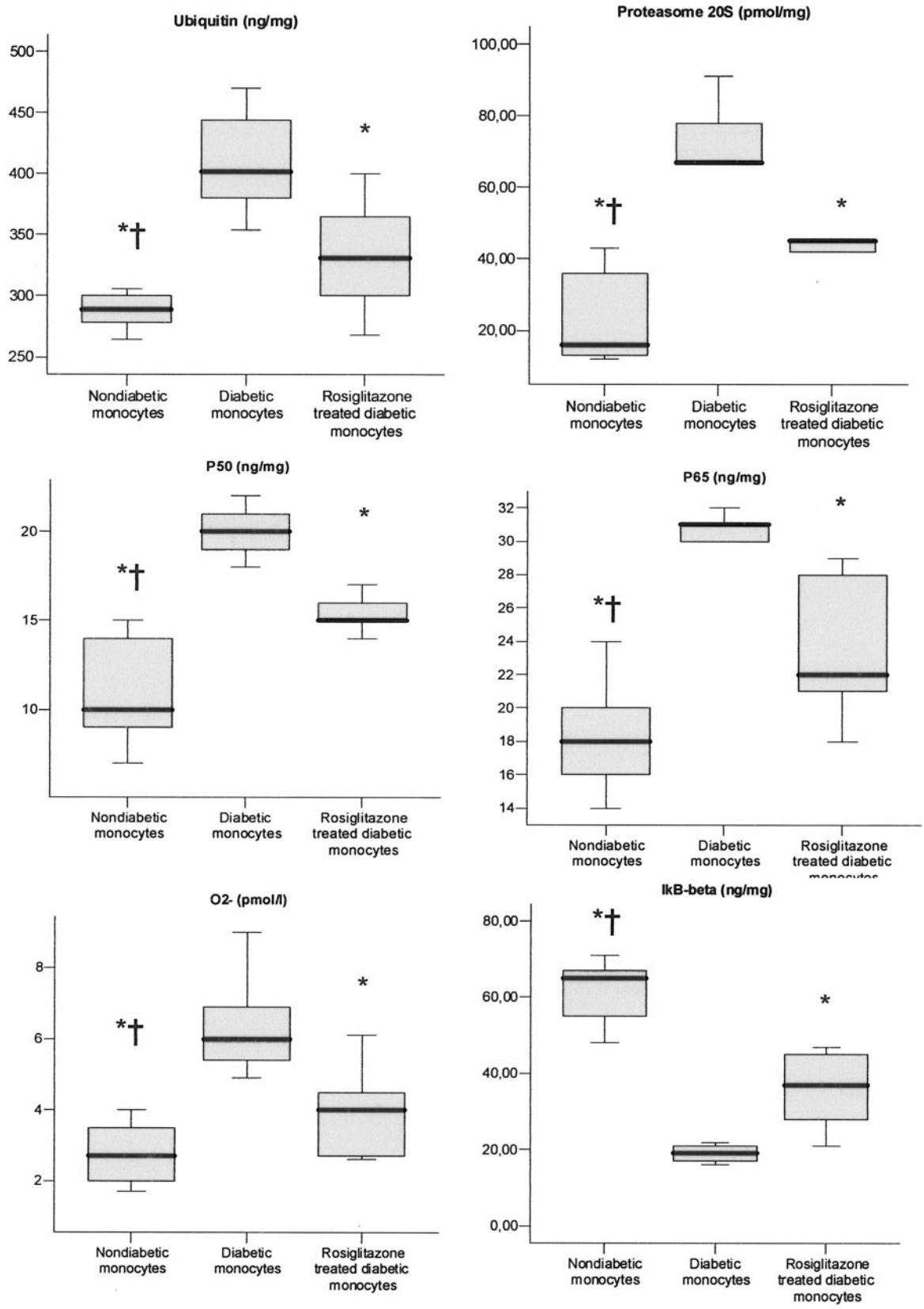

FIG. 6. Ubiquitin, proteasome 20S, activated NF-κB, and IκB-β in diabetic and nondiabetic monocytes. Purified diabetic monocytes were cultured in the presence or absence (48 h) of rosiglitazone (7.0 μmol/l). *$P < 0.05$ vs. the placebo group; †$P < 0.05$ vs. the rosiglitazone group.

mystery and requires further studies. On the other hand, the reduction in O_2^- production by monocytes after rosiglitazone treatment may allow a lower polyubiquitination, as evidenced by a reduction in ubiquitin levels. We therefore speculated that proteasome reduction by rosiglitazone may be induced by inhibition of oxidative stress

and polyubiquitination. Rosiglitazone treatment also produced a significant reduction in insulin-resistance, as indicated by reduced HOMA-IR levels. As insulin resistance is associated with increased cardiovascular and cerebrovascular risk (37), improved insulin sensitivity may be one mechanism by which rosiglitazone retards atherogenesis progression. On the other hand, oxidative stress has been shown to induce insulin resistance through NF-κB activation (38). In this perspective, rosiglitazone, by reducing oxidative stress and ubiquitin-proteasome activity, may enhance both insulin sensitivity and diabetic plaque stability. We also do not exclude the possibility that rosiglitazone inhibits NF-κB activation through additional mechanisms independent of PPAR-γ. Indeed, a PPAR-γ–independent pathway may be operative in neuronal cells via inhibition of inducible nitric oxide synthase (39). Furthermore, PPAR-γ agonists inhibit the nuclear translocation and subsequent DNA binding of NF-κB via an IκB–dependent pathway by inhibiting the immune response–induced degradation of IκBs (40). This provides evidence that a component of the anti-inflammatory effect is mediated by the action of PPAR-γ agonists on the systemic immune system. Although further investigation will be required to elucidate the upstream mechanisms by which PPAR-γ inhibits the degradation of IκBs, inhibition of cytokine gene expression suggests that activation of PPAR-γ may alter functional elements common to both of these pathways.

This study demonstrates enhanced ubiquitin-proteasome activity in diabetic atherosclerotic lesions, and it provides evidence that the activation of this system by inflammatory cells is associated with an NF-κB–dependent increase in inflammation, potentially promoting plaque rupture. Moreover, we hypothesize that thiazolodinediones may reduce NF-κB activation by modulation of ubiquitin-proteasome activity in human atherosclerotic lesions of diabetic patients.

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