C-reactive protein enhances macrophage lipoprotein lipase expression

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Abstract High serum levels of C-reactive protein (CRP), a strong predictor of cardiovascular events, are documented in patients with type 2 diabetes. Accumulating evidence suggests that CRP could directly promote arterial damage. To determine the role of CRP in diabetic atherosclerosis, we examined the effect of CRP on the expression of macrophage lipoprotein lipase (LPL), a proatherogenic molecule upregulated in type 2 diabetes. Treatment of human macrophages with native CRP increased, in a dose- and time-dependent manner, LPL protein expression and secretion. Modified CRP reproduced these effects. Preincubation of human macrophages with antioxidants, protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) inhibitors prevented CRP-induced LPL expression. Exposure of human macrophages to CRP further increased intracellular reactive oxygen species generation, classic PKC isozymes expression, and extracellular signal-regulated protein kinase 1/2 phosphorylation. In CRP-treated J774 macrophages, increased macrophage LPL mRNA levels and enhanced binding of nuclear proteins to the activated protein-1 (AP-1)-enhancing element were observed. These effects were prevented by antioxidants, as well as by PKC, MAPK, and AP-1 inhibitors. These data show for the first time that CRP directly increases macrophage LPL expression and secretion. Given the predominant role of macrophage LPL in atherogenesis, LPL might represent a novel factor underlying the adverse effect of CRP on the diabetic vasculature.—Maingrette, F., L. Li, and G. Renier. **C-reactive protein enhances macrophage lipoprotein lipase expression.** ***J. Lipid Res.*** **2008.** 49: **1926–1935.**

Supplementary key words atherosclerosis • type 2 diabetes • inflammation • oxidative stress • kinases

Since the hypothesis that inflammation might be an accomplice in the pathogenesis of atherosclerosis and type 2 diabetes was first proposed, many epidemiological and clinical studies have shown that circulating markers of inflammation predict the risk of future cardiovascular (CV) events and type 2 diabetes and that an ongoing acute phase response is present in patients with type 2 diabetes (1). In addition to being a CV risk marker (2, 3), C-reactive protein (CRP) could also make a direct contribution to the atherosclerotic process. Supporting this possibility, it has been shown that CRP is present in the plaque (4) and that exposure of cultured vascular cells to CRP leads to various proinflammatory and proatherogenic effects. Among the cell types found in the lesion, monocytes/macrophages have essential functions in all phases of atherosclerosis. Interaction of CRP with these cells induces an increase in the secretion of inflammatory mediators such as cytokines and chemokines (5, 6). Additional effects of CRP on monocytes/macrophages include induction of colony stimulating factor (CSF), matrix metalloproteinase (MMP), nitric oxide, and tumoricidal activity (7–10). Most importantly, CRP also appears to promote infiltration of monocytes into the vessel wall (11) and their subsequent development into foam cells (12).

Lipoprotein lipase (LPL) is a key enzyme in the hydrolysis of triglyceride-rich lipoproteins that is abundantly expressed by macrophages in the atherosclerotic lesion. Expression of macrophage-derived LPL in the arterial wall is proatherogenic (13, 14), probably via the enhancement of foam cell formation (15). We previously demonstrated that macrophage LPL is upregulated in patients with accelerated atherosclerosis, including type 2 diabetes (16–18), and have identified several macrophage LPL-stimulatory factors relevant to diabetes-related atherogenesis (19–23). Given the concomitant upregulation of CRP and macrophage LPL in human diabetes, we sought to investigate the regulation of macrophage LPL expression by CRP and the signaling pathways involved in this effect. Our data, which demonstrate a direct stimulatory effect of CRP on LPL secretion by cultured macrophages, suggest a new mechanism for the proatherogenic effect of CRP.

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RESEARCH DESIGN AND METHODS

Reagents

FCS was purchased from Wisent (St. Bruno, Quebec, Canada). RPMI 1640 medium, DMEM, Hank's balanced salt solution (HBSS), penicillin-streptomycin, glycine, SDS, and Trizol reagent were obtained from Invitrogen Life Technologies (Burlington, ON, Canada). Calphostin C, *N*-acetylcysteine (NAC), GF109203×, U0126, PD98059, BAY11-7085, curcumin, a nonspecific activated protein-1 (AP-1) inhibitor, and the pseudosubstrate myristoylated peptide inhibitor of protein kinase C (PKC)-α and -β (20–28) were purchased from Calbiochem (La Jolla, CA). Affinity-purified polyclonal antibody against extracellular signal-regulated protein kinase (ERK) 1/2 and antibodies against PKC-α, -β_I, -β_{II}, and -γ were bought from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-ERK1/2 antibody was obtained from Cell Signaling Technology (Pickering, ON, Canada). LY379196 was kindly provided by Eli Lilly (Indianapolis, IN). Monoclonal antibodies to human CD32 and CD64 were purchased from Lab Vision Corporation (Fremont, CA) and Ancell (Bayport, MN), respectively. Monoclonal antibody to human CD16 was kindly provided by Dr. Marika Sarfati, CHUM Research Center, Montreal, Canada. Vitamin E, E-TOXATE kit, sodium azide (NaN_3), D-glucose and 2′,7′-dichlorofluorescin diacetate (DCF-DA) were purchased from Sigma (St. Louis, MO). END-X B15 endotoxin removal affinity resin kit was obtained from Seikagaku America (Falmouth, MA).

CRP

Highly purified (>99%) recombinant human native CRP (nCRP) was purchased from Calbiochem. Purity of the nCRP preparation was confirmed by 12% SDS-PAGE. Endotoxin content of the recombinant CRP preparations was determined using the E-TOXATE kit. After removal of endotoxin using END-X B15 endotoxin removal affinity resin, nCRP was free of endotoxin, as assessed by the Limulus assay (<3 pg/ml). NaN_3 was removed from the commercial CRP preparation by dialysis against 500 ml 20 mmol/l Tris-HCl, pH 7.5, 140 mmol/l NaCl, 2 mmol $CaCl_2$ at 4°C. Modified CRP (mCRP) was prepared from nCRP by urea chelation. Briefly, nCRP was incubated with 8.0 M urea for 2 h at 37°C, then dialyzed against low-ionic-strength Tris-buffered saline.

Human macrophages

Human monocytes were isolated as previously described (23). Differentiation of monocytes into macrophages was achieved by culturing monocytes in RPMI 1640 medium supplemented with 1% (v/v) penicillin-streptomycin and 20% (v/v) autologous serum. The cells were incubated for 8 days at 37°C in a humidified 5% CO_2, 95% air atmosphere. At day 8, the medium of fully differentiated macrophages was changed to serum-free medium and cells were stimulated for different time periods with various concentrations of CRP.

Murine macrophages

The J774 murine macrophage cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and cultured in DMEM containing 10% FCS and 100 μg/ml penicillin-streptomycin.

Western blot analysis

LPL detection was achieved by immunoprecipitation followed by Western blot analysis as previously described (23). Briefly, after centrifugation of cell homogenates, the supernatant was collected and used for immunoprecipitation. Samples were incubated with the monoclonal anti-LPL 5D2 antibody (received from J. D. Brunzell, University of Washington, Seattle, WA) followed by incubation with anti-mouse IgG antiserum (Bio-Rad, Hercules, CA). The immunocomplexes were collected on protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) and washed. The pellet was resuspended in 1 × SDS loading buffer, and samples were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. Blots were incubated with the 5D2 antibody and then detected with HRP-conjugated donkey anti-mouse IgG antibody (1/5,000) followed by enhanced chemiluminescence. Band intensity was quantified by densitometry.

Measurement of ERK phosphorylation and classic PKC isoforms expression was performed by Western blot analysis using specific antibodies.

Analysis of LPL mRNA expression

Determination of LPL gene expression in J774 murine macrophages was performed by Northern blot analysis as described previously (23). After treatment, cells were lysed and total RNA was separated on a 1.2% agarose gel. mRNA expression was analyzed by hybridization with [^{32}P]dCTP-labeled LPL and human GAPDH cDNA probes.

DNA probes

The cDNA probe for detection of murine LPL was prepared by the PCR technique (23). The cDNA probe for murine GAPDH was purchased from ATCC. A 20 mer double-stranded oligonucleotide (5′-GGGCACCTGACTAAGGCCAG-3′; 5′-TGTGCTGGCCTTAGTCAGGT-3′) containing the consensus sequence for the AP-1-responsive element of the murine LPL gene promoter was synthesized. After annealing, the double-stranded oligonucleotide was labeled with [γ-^{32}P]ATP by using the Boehringer Mannheim 5′ end-labeling kit (Indianapolis, IN).

DNA binding assay

Isolation of the nuclei was performed as described previously (23). After lysis of the nuclei, aliquots of the supernatants were frozen at −80°C, and protein concentration was determined. DNA retardation (mobility shift) electrophoresis assay was performed as described previously (23).

Measurement of intracellular reactive oxygen species generation

Human monocytes were plated at a density of 5×10^5/well in a 96 MicroWell Nunclon Δ sterile optical bottom plate (Nunc, Rochester, NY) in RPMI 1640 medium supplemented with 1% (v/v) penicillin-streptomycin and 20% (v/v) autologous serum. The cells were incubated for 8 days at 37°C in a humidified 5% CO_2, 95% air atmosphere. The culture medium was changed at days 4 and 8. At day 8, fully differentiated macrophages were treated with 3 μg/ml CRP for 1 h, then washed prior to the addition of the cell-permeable fluorogenic DCF-DA (10 μmol/l) probe for 30 min. At the end of this incubation period, cells were washed with cold PBS, then fixed with 2% paraformaldehyde. Intracellular reactive oxygen species (ROS) generation was quickly monitored by measuring fluorescence, using excitation and emission wavelengths of 498 nm and 522 nm, respectively.

Determination of extracellular LPL activity

One hour before the end of the incubation period, 50 U/ml heparin was added to the medium. Extracellular LPL activity was determined using the Confluolip kit (Progen, Heidelberg, Germany). Levels of macrophage LPL activity were normalized to levels of total cell proteins.

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Determination of cell viability

Cell viability after treatment with CRP and pharmacological inhibitors was assessed by trypan blue exclusion and was consistently found to be >90%.

Determination of total protein concentration

Total protein content was estimated according to the Bradford method using a colorimetric assay (Bio-Rad, Mississauga, ON, Canada).

Statistical analysis

Data presented were generated using blood obtained from different donors. All values were expressed as means ± SEM. Data were analyzed by one-way ANOVA, followed by the Tukey test. A P value of <0.05 was considered statistically significant.

RESULTS

Effect of nCRP on human macrophage LPL protein expression and secretion

Incubation of macrophages with nCRP (0–5 μg/ml) for 24 h increased, in a dose-dependent manner, LPL protein expression (**Fig. 1A**). LPL induction was evident at 1 μg/ml and reached a maximum at 3 μg/ml nCRP (Fig. 1A) No further increase in macrophage LPL expression was noticed with nCRP concentrations exceeding 5 μg/ml [LPL protein levels (% of control values): nCRP (5 μg/ml): 180 ± 20, $P < 0.001$; nCRP (10 μg/ml): 180 ± 16, $P < 0.001$; nCRP (25 μg/ml): 160 ± 8, $P < 0.001$; nCRP (50 μg/ml): 173 ± 12, $P < 0.001$)]. Both nCRP and dialyzed CRP preparations showed equivalent ability to induce macrophage LPL protein expression, whereas NaN_3 alone, at a concentration equivalent to 3 μg/ml nCRP (0.00015%), did not affect this parameter (data not shown). Induction of macrophage LPL by nCRP (3 μg/ml) was time-dependent, observed from 12 h to 72 h with maximal effect at 24 h (Fig. 1B). Recovery of enhanced heparin-releasable LPL activity reflected the increase in intracellular LPL protein levels in nCRP-treated human macrophages (Fig. 1C). Induction of LPL activity was prevented by heat inactivation of CRP (Fig. 1C).

To compare the biological effects of nCRP and mCRP, LPL protein expression was determined in macrophages treated for 6 h to 24 h with 3 μg/ml nCRP or mCRP. Although both CRP preparations significantly increased macrophage LPL protein expression at 12 and 24 h, mCRP was more potent at 12 h (**Fig. 2A**). In contrast, nCRP and mCRP did not differ in their ability to induce LPL secretion at 12 h and 24 h (Fig. 2B).

CRP effect on human macrophage LPL protein expression is mediated by FcγRII receptors

Because CRP binds to FcγRI and FcγRII receptors on vascular cells, we next evaluated whether CRP binding to these receptors is involved in the stimulatory effect of CRP on macrophage LPL expression. Pretreatment of human macrophages for 1 h with antibody to CD32 prevented the effect of nCRP on macrophage LPL protein expression, whereas preincubation of the cells with antibody to CD64

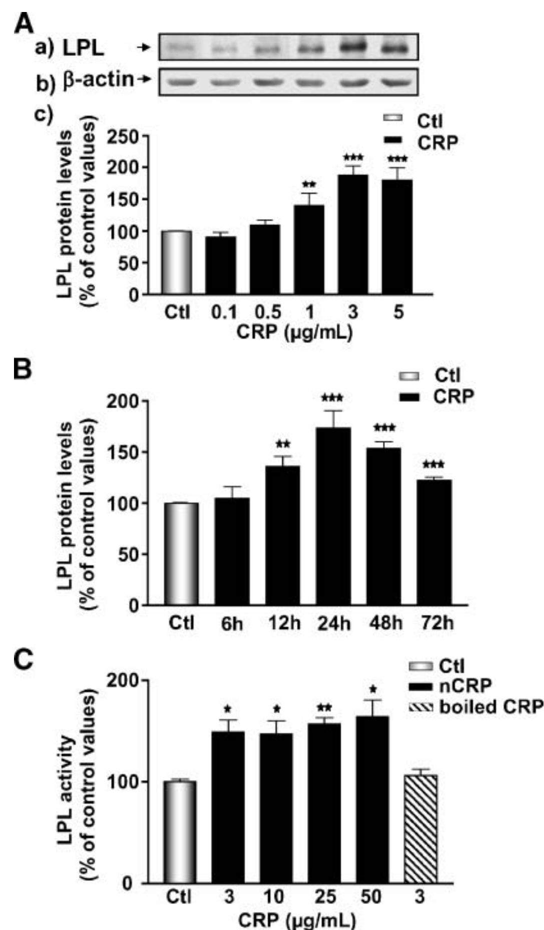

Fig. 1. Effect of recombinant human native C-reactive protein (nCRP) on human macrophage lipoprotein lipase (LPL) protein expression and secretion. Human macrophages were incubated for 24 h with endotoxin-free nCRP (0–5 μg/ml) (A) or for 6 h to 72 h with 3 μg/ml nCRP (B). At the end of these incubation periods, cells were lysed and LPL protein expression was determined by Western blot analysis (top). LPL protein levels were normalized to the levels of β-actin protein. C: Human macrophages were incubated for 24 h with nCRP (0–50 μg/ml) or boiled CRP (3 μg/ml). Extracellular LPL activity was determined as described in Research Design and Methods. Data are means ± SEM of four different experiments. *$P < 0.05$ vs. control; **$P < 0.01$ vs. control; ***$P < 0.001$ vs. control. a, LPL protein levels; b, beta- actin levels; c, LPL protein levels normalized to beta-actin levels.

had no effect (**Fig. 3**). Addition of the irrelevant murine anti-IgG_1 antibody did not affect this parameter [LPL protein levels (% of control values): anti-IgG_1 antibody (10 μg/ml): 117 ± 17, $P > 0.05$]. An anti-CD16, but not an anti-CD32 antibody, reduced the mCRP effects [extracellular LPL activity (% of control value): mCRP (3 μg/ml): 150 ± 5, $P < 0.001$; anti-CD16 (5 μg/ml) + mCRP: 116 ± 3; anti-CD32 + mCRP: 143 ± 5, $P < 0.01$].

Role of oxidative stress and PKC on CRP-stimulated human macrophage LPL protein expression

Exposure of human macrophages to CRP (3 μg/ml) led to a significant increase of intracellular ROS generation, as assessed by DCF-DA (**Fig. 4A**). Pretreatment of the cells with vitamin E (50 μmol/l), NAC (10 mmol/l), or an anti-

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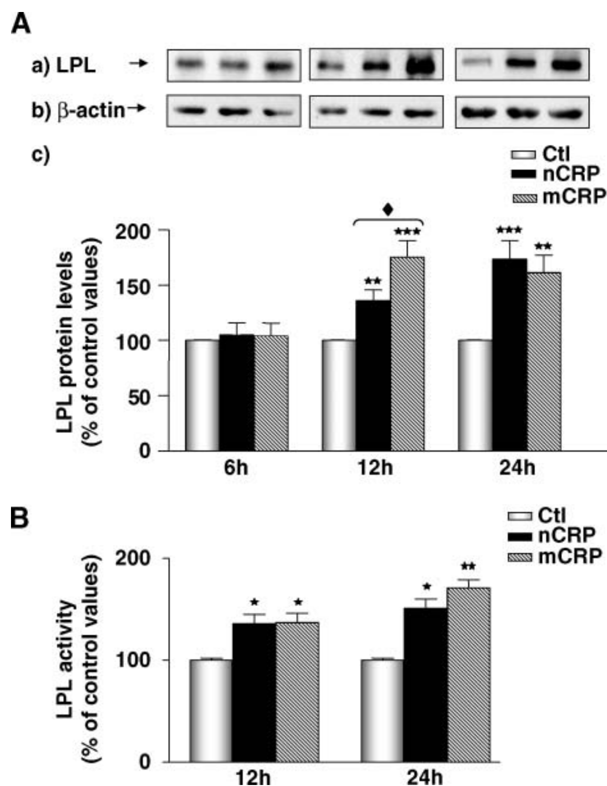

Fig. 2. Effect of nCRP vs. modified CRP (mCRP) on human macrophage LPL protein expression and secretion. A: Human macrophages were incubated for 6 h to 24 h with nCRP or mCRP (3 μg/ml). At the end of these incubation periods, cells were lysed and LPL protein expression was determined by Western blot analysis (top). LPL protein levels were normalized to the levels of β-actin protein. B: Human macrophages were incubated for 12 to 24 h with nCRP or mCRP (3 μg/ml). Extracellular LPL activity was determined as described in Research Design and Methods. Data are means ± SEM of four different experiments. * $P < 0.05$ vs. control; ** $P < 0.01$ vs. control; *** $P < 0.001$ vs. control; ◆ $P < 0.05$ vs. nCRP. a, LPL protein levels; b, beta- actin levels; c, LPL protein levels normalized to beta-actin levels.

body to CD32 (5 μg/ml) abolished this effect (Fig. 4A). Exposure of unstimulated cells to these compounds did not affect basal DCF fluorescence (data not shown). Incubation of human macrophages with vitamin E (50 μmol/l) or NAC (10 mmol/l) prevented the stimulatory effect of CRP on LPL protein expression (Fig. 4B). These compounds and the NADPH oxidase inhibitor apocynin (10 μmol/l) also abolished the effect of CRP on LPL secretion (Fig. 4C).

To examine the involvement of the PKC pathway in the effect of CRP on macrophage LPL expression, total cell lysates were analyzed for the presence of conventional PKC isoforms. Incubation of macrophages with CRP (3 μg/ml) for 10 min increased the expression of PKC-α, -β_I, -β_{II}, and -γ by 2.7-, 2.2-, 1.9-, and 1.8-fold, respectively (**Fig. 5A**). Next, the effect of the pan-specific and conventional PKC inhibitors calphostin C (0.1 μg/ml) and GF109203× (20 nmol/l) on CRP-induced LPL expression was determined. Pretreatment of macrophages with these compounds totally abolished the stimulatory effect of CRP on macrophage LPL protein expression (Fig. 5B) and secretion (Fig. 5C). Inhibition of CRP-induced LPL secretion was further observed following incubation of macrophages with the myristoylated peptide inhibitor of PKC-α and -β (50 μmol/l) and the PKC-β inhibitor LY379196 (20 nmol/l) (Fig. 5C).

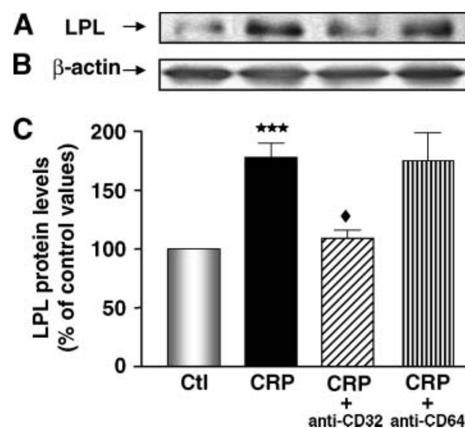

Fig. 3. Role of FcγRII receptor on CRP-induced human macrophage LPL protein expression. Human macrophages were preincubated for 1 h with antibody against CD32 (5 μg/ml) or CD64 (5 μg/ml) and then incubated for 24 h with 3 μg/ml CRP. At the end of this incubation period, cells were lysed and LPL protein expression was determined by Western blot analysis (top). LPL protein levels were normalized to the levels of β-actin protein. Data are means ± SEM of four different experiments. *** $P < 0.001$ vs. control; ◆ $P < 0.05$ vs. CRP. a, LPL protein levels; b, beta- actin levels; c, LPL protein levels normalized to beta-actin levels.

Role of ERK in CRP-induced human macrophage LPL protein expression

To determine the role of the ERK pathway in the regulation of macrophage LPL by CRP, human macrophages were incubated for 1 h with PD98059 (10 μmol/l) or U0126 (20 μmol/l) prior exposure to CRP. Pretreatment of the cells with these inhibitors totally abolished CRP-induced macrophage LPL protein expression and secretion (**Fig. 6A, B**). Exposure of unstimulated cells to PD98059 and U0126 did not affect basal LPL protein levels or activity (data not shown). To further ascertain the involvement of the ERK1/2 pathway in CRP-treated macrophages, these cells were treated with CRP (3 μg/ml) for 0–30 min, and the expression level of phosphospecific ERK1/2 was determined by immunoblot analysis. Treatment of macrophages with CRP induced ERK1/2 phosphorylation in a time-dependent manner, with peak activation occurring at 10 min. This effect was completely abrogated by prior incubation of the cells with calphostin C or GF109203× (Fig. 6C). Under these conditions, no modulation of total cellular ERK, used as control, was observed (Fig. 6C).

Effect of glucose on CRP-induced macrophage LPL secretion

The effect of CRP on LPL secretion was further studied in high-glucose-treated human macrophages. As reported previously (22), incubation of macrophages in a high-

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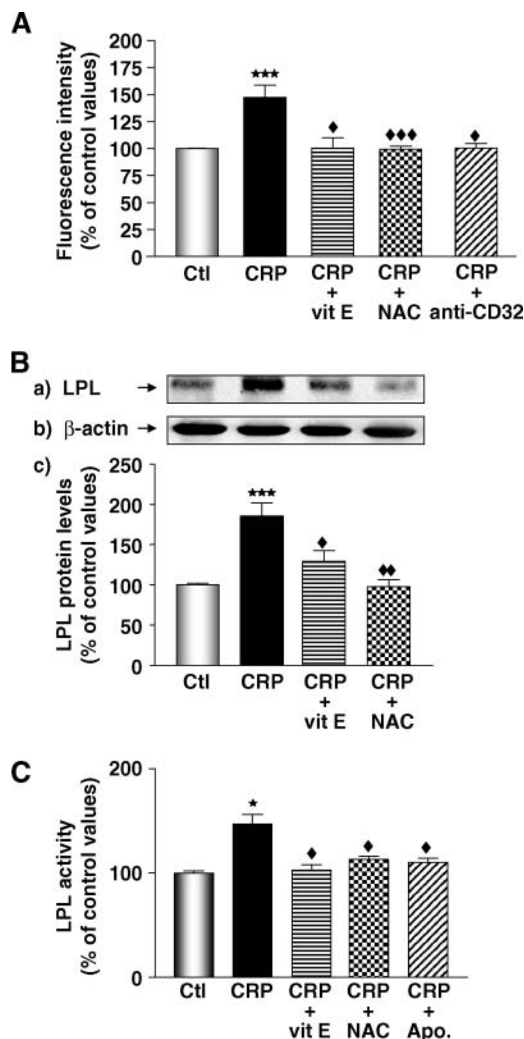

Fig. 4. Role of oxidative stress on CRP-stimulated human macrophage LPL expression and secretion. A: Human macrophages were preincubated for 1 h with vitamin E (50 μmol/l), *N*-acetylcysteine (NAC) (10 mmol/l), or an antibody against CD32 (5 μg/ml) then treated for 1 h with 3 μg/ml CRP, and intracellular reactive oxygen species generation was measured. Results are reported as percent of control values. B: Human macrophages were preincubated for 1 h with vitamin E (50 μmol/l) or NAC (10 mmol/l) prior to exposure to CRP (3 μg/ml) for 24 h. At the end of this incubation period, cells were lysed and LPL protein expression was determined by Western blot analysis. LPL protein levels were normalized to the levels of β-actin protein. C: Human macrophages were preincubated for 1 h with vitamin E (50 μmol/l), NAC (10 mmol/l), or apocynin (10 μmol/l) then treated for 1 h with 3 μg/ml CRP. Extracellular LPL activity was determined as described in Research Design and Methods. Data are means ± SEM of four different experiments. * $P < 0.05$ vs. control; *** $P < 0.001$ vs. control; ◆ $P < 0.05$ vs. CRP; ◆◆ $P < 0.01$ vs. CRP; ◆◆◆ $P < 0.001$ vs. CRP. a, LPL protein levels; b, beta- actin levels; c, LPL protein levels normalized to beta-actin levels.

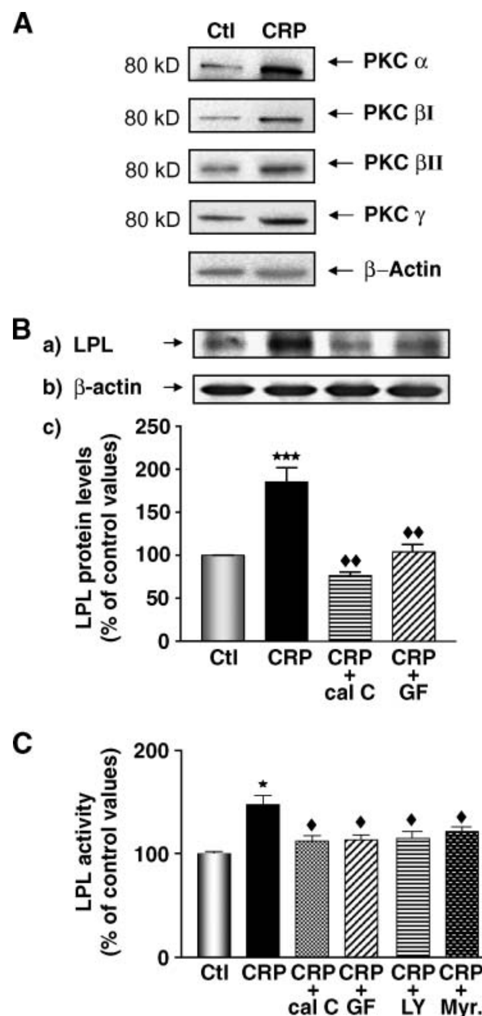

Fig. 5. Role of protein kinase C (PKC) on CRP-stimulated human macrophage LPL expression and secretion. A: Human macrophages were incubated with 3 μg/ml CRP for 10 min. Whole-cell protein extracts were assayed for conventional PKC isoforms. Figure 4A illustrates the results of one representative experiment out of three. B: Human macrophages were preincubated for 1 h with calphostin C (cal C) (0.1 μg/ml) or GF109203× (GF) (20 nmol/l) prior to incubation with 3 μg/ml CRP for 24 h. At the end of this incubation period, cells were lysed and LPL protein expression was determined by Western blot analysis. LPL protein levels were normalized to the levels of β-actin protein. C: Human macrophages were preincubated for 1 h with calphostin C (0.1 μg/ml), GF109203× (20 nmol/l), LY379196 (LY) (20 nmol/l), or the myristoylated peptide inhibitor of PKCα/β (Myr) (50 μmol/l) prior to incubation with 3 μg/ml CRP for 24 h. Extracellular LPL activity was determined as described in Research Design and Methods. Data are means ± SEM of four different experiments. * $P < 0.05$ vs. control; *** $P < 0.001$ vs. control; ◆ $P < 0.05$ vs. CRP; ◆◆ $P < 0.01$ vs. CRP. a, LPL protein levels; b, beta- actin levels; c, LPL protein levels normalized to beta-actin levels.

glucose environment led to increased macrophage LPL expression. Addition of CRP did not further increase LPL secretion under hyperglycemic conditions [extracellular LPL activity (% of control value): CRP (3 μg/ml): 140 ± 6, $P < 0.05$; glucose (20 mM): 140 ± 6, $P < 0.05$; CRP + glucose: 147 ± 8, $P < 0.05$].

Effect of CRP on murine macrophage LPL expression

Role of oxidative stress, PKC, and mitogen-activated protein kinase. Given the limited amount of biological material that can be obtained from human macrophages, the molecular mechanisms involved in CRP-induced LPL expression were studied in the J774 murine macrophage cell line. To ascer-

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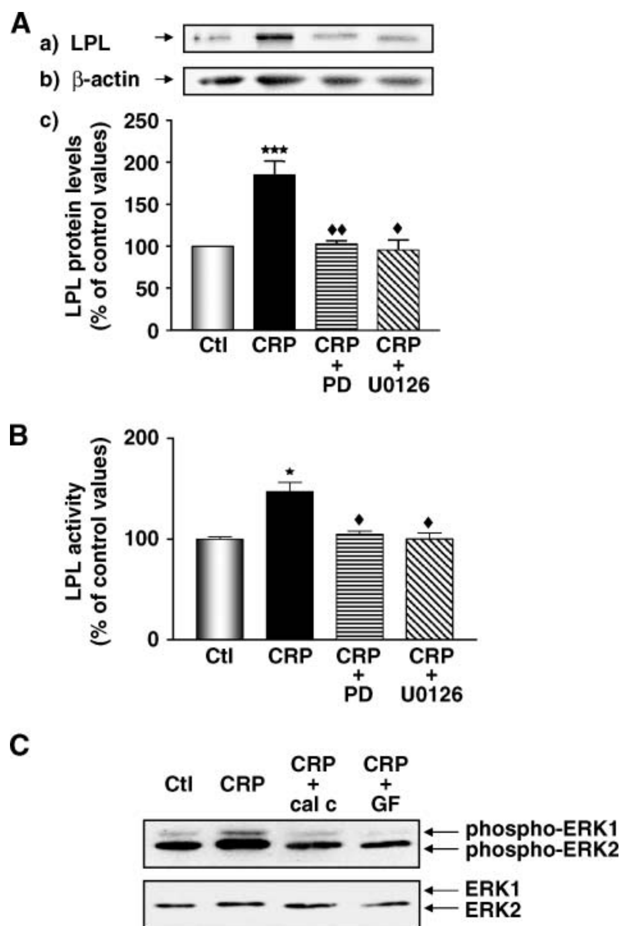

Fig. 6. Role of MAPK on CRP-induced human macrophage LPL expression and secretion. Human macrophages were preincubated for 1 h with PD98059 (PD) (10 μmol/l) or U0126 (20 μmol/l), then treated for 24 h with 3 μg/ml CRP. At the end of this incubation period, LPL protein expression (A) and extracellular LPL activity (B) were determined. Data are means ± SEM of four different experiments. * $P < 0.05$ vs control; *** $P < 0.001$ vs. control; ♦ $P < 0.05$ vs. CRP, ♦♦ $P < 0.01$ vs. CRP. C: Human macrophages were preincubated for 1 h with calphostin C (cal C) (0.1 μg/ml) or GF109203× (GF) (20 nmol/l) then treated for 10 min with 3 μg/ml CRP. Extracellular signal-regulated protein kinase (ERK) phosphorylation was assessed by Western blot using phospho-specific or specific ERK1/2 antibodies. One representative blot is shown. a, LPL protein levels; b, beta- actin levels; c, LPL protein levels normalized to beta-actin levels.

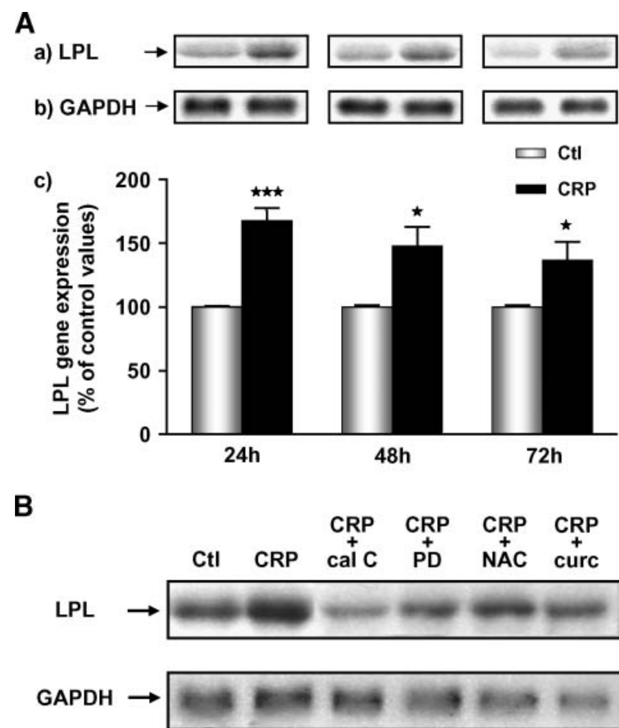

Fig. 7. Effect of CRP on murine macrophage LPL gene expression. Role of oxidative stress, PKC, and MAPK. A: J774 cells were incubated for 24 h to 72 h with 3 μg/ml CRP. At the end of these incubation periods, cells were lysed and total RNA was extracted and analyzed by Northern blot analysis for LPL (a) and GAPDH (b) mRNA expression. Levels of LPL mRNA were normalized to the levels of GAPDH mRNA (c). Results are means ± SEM of four different experiments, * $P < 0.05$ vs. control; *** $P < 0.001$ vs. control. B: J774 cells were preincubated for 1 h in the presence of calphostin C (cal C) (0.1 μg/ml), PD98059 (PD) (10 μmol/l), NAC (10 mmol/l) or curcumin (curc) (10 μmol/l), then treated for 24 h with 3 μg/ml CRP. At the end of these incubation periods, cells were lysed and total RNA was extracted and analyzed by Northern blot analysis for LPL (upper panel) and GAPDH (lower panel) mRNA expression. One representative blot out of three independent experiments is shown.

tain the validity of this model, LPL protein and gene expression were first measured in CRP-treated J774 macrophages. Incubation of murine macrophages with native CRP (3 μg/ml) for 24 h significantly increased macrophage LPL protein expression [LPL protein levels (% increase over control values): CRP (3 μg/ml): 210 ± 22, $P < 0.001$]. Stimulation of J774 macrophages for 24 h with 3 μg/ml CRP also significantly increased LPL mRNA expression in these cells. This effect was sustained up to 72 h (**Fig. 7A**). Under these experimental conditions, no modulation of GAPDH, used as an internal control, was observed (Fig. 7A). Pretreatment of murine macrophages with NAC, PKC, mitogen-activated protein kinase (MAPK), and AP-1 inhibitors abolished the stimulatory effect of CRP on macrophage LPL gene expression (Fig. 7B).

Effect of CRP on the binding of nuclear proteins to the AP-1 sequence of the murine LPL gene promoter

Finally, to determine whether incubation of macrophages with CRP could result in changes at the level of LPL gene binding proteins, the binding activity of nuclear proteins extracted from CRP-treated J774 macrophages to the AP-1 consensus sequence of the LPL gene promoter was analyzed. Electrophoretic mobility shift assay showed that a 24 h incubation time of these cells with CRP led to an increased binding of nuclear proteins to the AP-1 regulatory sequence of the LPL gene promoter. This binding complex was specifically competed in the presence of a 1,000-fold molar excess of the unlabeled AP-1 oligonucleotide. Pretreatment with PKC, MAPK, and AP-1 inhibitors, as well as with the antioxidant NAC, prevented the enhanced binding of nuclear proteins to AP-1 (**Fig. 8**).

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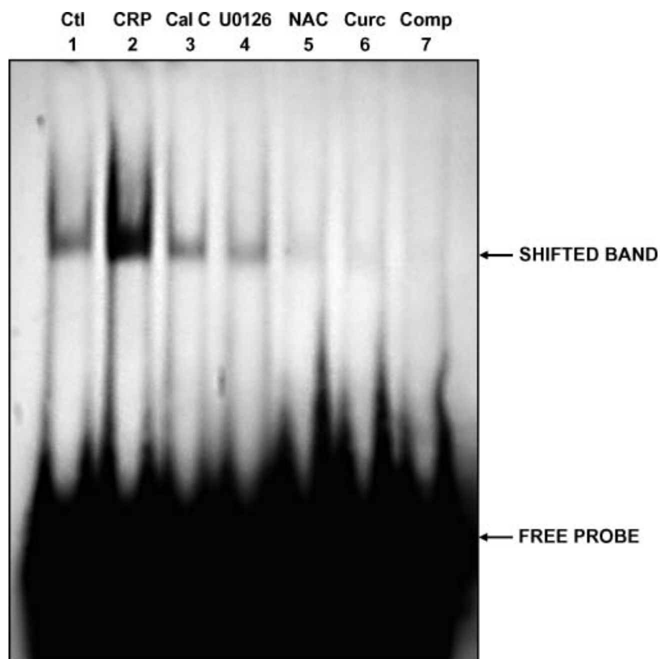

Fig. 8. Effect of CRP on the binding of nuclear proteins extracted from J774 cells to the regulatory activated protein-1 (AP-1) sequence of the LPL gene promoter. J774 cells were incubated with 3 μg/ml CRP for 24 h in the presence or absence of calphostin C (Cal C) (0.1 μg/ml), U0126 (20 μmol/l), NAC (10 mmol/l), or curcumin (curc) (10 μmol/l). Nuclear proteins isolated from these cells were incubated with the double-stranded AP-1 regulatory element of the LPL gene promoter. Retardation was assessed by gel electrophoresis in 4% PAGE. Figure shows one representative experiment out of three. Lane 1: untreated cells (Ctl); lane 2: CRP-treated cells (CRP); lane 3: CRP + calphostin C (Cal C); lane 4: CRP + U0126 (U0126); lane 5: CRP + NAC (NAC); lane 6: CRP + curcumin (Curc); lane 7: competition of the CRP-induced binding complex in the presence of 1,000-fold molar excess of the unlabeled AP-1 oligonucleotide (Comp).

DISCUSSION

Because of its ability to promote foam cell formation (15), macrophage LPL has been identified as a potential target for intervention and its regulation has been the subject of extensive investigation. We have previously demonstrated that macrophage LPL is upregulated in patients with type 2 diabetes (18) and have proposed a role for peripheral factors, including glucose, in this alteration (19–23).

In addition to being a predictor of CV risk in patients with type 2 diabetes (24), CRP may also contribute to the development of atherosclerosis. Arguing for this hypothesis, CRP has been reported to stimulate vascular cells to secrete a vast array of inflammatory factors (25).

Data presented here demonstrate for the first time that CRP, at concentrations associated with increased CV risk (26) and commonly observed in diabetic patients (27), enhances the expression of macrophage LPL. This effect remained unchanged by hyperglycemia, suggesting that CRP and glucose operate through common molecular mechanisms to induce LPL. Because endotoxin is a well-known macrophage LPL inhibitory factor (28), endotoxin contamination of the CRP preparation may not account for this effect. Our findings that low CRP concentrations induce optimal macrophage LPL expression compare well with the results of previous studies showing that a triggering signal provided by 1 to 10 μg/ml CRP induces maximal macrophage tumoricidal activity, MMP-9 expression, and CSF and interleukin-1α production (5, 7, 8, 10).

It has been previously suggested that conformational changes in CRP are required for expression of biological functions (29, 30). Our data, which demonstrate that mCRP induces LPL protein expression at 12 h to a greater extent than does nCRP, underscore the potential biological significance of mCRP in the regulation of macrophage LPL expression. These results, along with our observation that both forms of CRP are equally effective in stimulating macrophage LPL at 24 h, support the notion that CRP effects on macrophage LPL might depend, at least in part, on the conversion of nCRP to mCRP. Although this may be true, nCRP may also independently regulate macrophage LPL expression. Supporting this possibility, we found that both forms of CRP stimulate macrophage LPL activity at 12 h and 24 h to the same extent. The relevance of mCRP to atherosclerosis is unknown. Although its presence in lesions is still disputed, in vivo and in vitro studies have substantiated discordant effects of mCRP in atherogenesis (29–31). Whether these opposing results stem, at least in part, from differences in the long-term versus acute effect of the protein remains to be clarified.

In contrast to mCRP, nCRP has been identified in the arterial plaque (4), where it colocalizes with macrophage-derived foam cells (32). Because vessel wall LPL is proatherogenic and is involved in foam cell formation, induction of macrophage LPL by nCRP may represent a new molecular mechanism by which this protein induces proatherogenic effects and favors macrophage lipoprotein uptake.

Several lines of evidence suggest that CRP concentrations in the atherosclerotic lesion might exceed serum levels. Hence, it was important to assess the regulatory effect of CRP concentrations exceeding CV risk on macrophage LPL expression. Our finding that CRP, at concentrations ranging from 10 μg/ml to 50 μg/ml, significantly enhances macrophage LPL expression and activity in vitro supports the relevance of our observations to atherogenesis.

Many studies have demonstrated the presence of CRP receptors on mononuclear cells. Although nCRP can bind FcγRI (CD64) receptors on human monocytes (33), FcγRII (CD32) has been shown to be the major receptor for nCRP on these cells (34). Consistent with these findings, our results demonstrate that preincubation of human macrophages with FcγRII but not FcγRI antibody prevented nCRP-induced macrophage LPL protein expression. In contrast to nCRP, the actions of mCRP appear to be mediated predominantly through CD16 (29, 30). Consistent with this possibility, our data indicate that induction of LPL by CRP is reduced by an anti-CD16 antibody. Because this antibody did not produce complete reversal of mCRP effects, other as-yet-unidentified cell surface receptors might be involved.

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Macrophage LPL induction in response to CRP appears to be exerted at the transcriptional level, as reflected by the parallel increase in LPL gene and protein expression. Transcriptional activation of the LPL gene by CRP may theoretically involve AP-1. Indeed, an AP-1-responsive element has been located in the regulatory sequence of the LPL gene (35), and increased AP-1 activation has been documented in CRP-treated vascular smooth muscle cells (36). Supporting this hypothesis, our results demonstrate that curcumin, a nonspecific inhibitor of the transcriptional activity of AP-1, inhibits CRP-induced macrophage LPL mRNA levels and that CRP stimulates nuclear protein binding to the AP-1 sequence of the LPL gene. Although these observations would suggest the involvement of AP-1 in the induction of macrophage LPL by CRP, further studies using a molecular-dominant strategy are needed to confirm this possibility. These data, along with our previous observations showing the involvement of AP-1 in the induction of macrophage LPL by several factors dysregulated in diabetes (19, 20, 22, 23), further stress the key role of this transcription factor in the regulation of macrophage LPL gene expression in human diabetes.

Human diabetes represents a state of heightened oxidative stress, and oxidative events are known to be closely related to inflammation. Evidence has been provided that functional activation of CD32 by CRP is linked to ROS production and proinflammatory effects in vascular cells (37, 38) and that macrophage LPL expression is upregulated by oxidative stress (39). In addition, a role for oxidative stress in the inhibitory effect of CRP on macrophage cholesterol efflux has been recently demonstrated (40). Consistent with a key role of oxidative stress in the regulatory effect of CRP on macrophage LPL, our results demonstrate that CRP increased intracellular ROS generation in human macrophages and that antioxidants prevented the stimulatory effect of CRP on macrophage LPL expression. NADPH oxidase is a major source of ROS in vascular cells and is upregulated in CRP-treated THP-1 foam-like cells (40). Our observation that incubation of human macrophages with apocynin blocks CRP-induced LPL secretion supports a role for NADPH oxidase in this effect.

PKC and MAPK are implicated in the pathogenesis of diabetic vasculopathies, and ROS are well-known activators of these kinases. We have previously demonstrated that PKC activation and PKC-dependent MAPK activation are major participants in the regulation of macrophage LPL (19, 20, 22, 23). Although indirect evidence has been provided for a role of PKC in CRP-induced platelet adhesion and VCAM-1 expression (41, 42), the levels of PKC activity in CRP-treated vascular cells has not yet been documented. In the present study, we demonstrated that CRP increases the expression of conventional PKC isoforms in macrophages. Our results showing that induction of LPL by CRP can be blocked by a pseudosubstrate PKCα/β inhibitor and a PKCβ inhibitor, LY379196, support a role for PKCβ as a signaling molecule mediating the stimulatory effect of CRP on macrophage LPL. Numerous studies have shown that MAPK, and particularly ERK1/2, are implicated in the activation of vascular cells by CRP (36, 43–48). In accordance with previous studies showing a rapid activation of ERK1/2 after CRP stimulation of endothelial cells (48), we found that CRP induced ERK1/2 phosphorylation in macrophages. That inhibition of the ERK pathway totally abolished CRP-induced macrophage LPL expression, at both the gene and protein levels, indicates that activation of these kinases is required for the stimulatory effect of CRP on macrophage LPL. Several lines of evidence indicate that PKC activates the MAPK signal transduction (49, 50). Consistent with these results, we found that PKC inhibition abolished CRP-induced ERK1/2 activation in macrophages, thus identifying MAPK as a downstream target of PKC in these cells. Previous studies have demonstrated that PKC and MAPK can act as mediators of AP-1 activation (51, 52). On the basis of our results showing that antioxidants, as well as PKC/MAPK inhibitors, prevented the activation of the AP-1 transcription factor by CRP, we propose a model in which CRP, by increasing ROS production, leads to the activation of PKC/MAPK pathways and the subsequent activation of AP-1 in macrophages.

A vast literature has been generated in the last few years, and it all points toward the critical role that CRP plays in all stages of CV disease. The role of CRP has evolved from just a marker of CV risk to an active player in the atherosclerotic process. Even though this latter role is still being questioned (53, 54), our results add weight to the growing number of studies showing that CRP can directly activate vascular cells, and further corroborate a role for CRP as an active player in the promotion of atherogenesis.

In conclusion, this study demonstrates for the first time that CRP stimulates macrophage LPL expression and secretion in vitro. This effect is mediated through oxidative stress and appears to involve PKC and MAPK activation. These data suggest a new mechanism by which CRP may promote atherogenesis. Establishing whether the in vivo effect of CRP on macrophage function favors atherogenesis in humans is of clinical interest, especially in patients with diabetes, who have increased serum CRP levels and demonstrate enhanced secretion of proatherogenic cytokines by macrophages, including LPL.

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