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C-Reactive Protein Promotes Adhesion of Monocytes to Endothelial Cells Via NADPH Oxidase-Mediated Oxidative Stress

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ABSTRACT

Enhanced monocyte adhesion to endothelial cells is an early event in atherogenesis. It has been shown that C-reactive protein (CRP) plays a key role in atherogenesis. Here, we investigated the effects of CRP on monocyte-endothelial cell adhesion and tested the hypothesis that NADPH oxidase (NOX)-mediated oxidative stress might play a key role in CRP-induced monocyte-endothelial cell adhesion. Firstly, 36 patients with carotid intima-media thickness (IMT) incrassation and 34 controls were enrolled in this study. The levels of glucose, lipids, CRP, monocyte chemotractant protein (MCP-1), malondialdehyde (MDA), and protein carbonylation were analyzed. The results showed that carotid IMT was associated with abnormal lipid metabolism, including elevated CRP, triglycerides (TG) ($P < 0.01$) and decreased high density lipoprotein (HDL) level ($P < 0.05$). The levels of CRP and MCP-1 in patients with carotid IMT incrassation were increased compared with the controls ($P < 0.01$). Moreover, patients with carotid IMT incrassation displayed enhanced MDA and protein carbonylation levels ($P < 0.01$), accompanied by activation and up-regulation of NOX in monocytes ($P < 0.05$) compared with the controls. The monocytes isolated from five healthy donors were used for in vitro experiments. Reactive oxygen species (ROS) production and NOX expression in monocytes were examined. The results also indicated that CRP could promote the adhesion of monocyte-endothelial cell by up-regulation of MCP-1 expression ($P < 0.05$). Importantly, NFκ B and p38 MAPK signaling pathways, which were activated by NOX-derived ROS, were involved in CRP-induced monocyte-endothelial cell adhesion and up-regulation of MCP-1 expression. These data suggested that CRP could promote the adhesion of monocytes to endothelial cells via NOX-mediated oxidative stress. J. Cell. Biochem. 113:857–867, 2012.

KEY WORDS: C-REACTIVE PROTEIN; INTIMA-MEDIA THICKNESS; NADPH OXIDASE; MONOCYTES

Atherosclerosis was considered to be a simple disease involving arterial lipid accumulation previously, and now it is known to involve a defined cascade of inflammatory processes. It was suggested that the inflammation occurred simultaneously with lipid accumulation in the artery wall in animal models and patients with atherosclerosis.

Carotid intima-media thickness (IMT) incrassation occurs in the onset of atherosclerosis. It represents a pathological and physiological status of the vascular wall and it is a useful biomarker for atherosclerotic vascular disease. During the course of carotid IMT incrassation, recruitment and adhesion of monocytes to the early atherosclerotic lesion is a critical earlier event [Hansson et al., 2006; Barton et al., 2007; Libby et al., 2010; Nakagomi et al., 2010]. The monocyte-endothelial interaction consists of well organized sequential events, such as rolling, adhesion, and transmigrations [Butcher, 1991]. monocyte chemotractant protein (MCP-1), a major regulator of monocyte trafficking, plays a distinct role in these processes [Gerszten et al., 1999]. Previous study has shown that

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MCP-1 deficiency abolished the adhesion and infiltration of monocytes, and delayed progression to atherosclerosis [Kuziel et al., 1997]. MCP-1 stimulates the recruitment of monocytes to the site of injury via activation of integrins and thus promotes migration into the vessel wall [Reape and Groot, 1999; Libby et al., 2010].

C-reactive protein (CRP) had been regarded as a traditional nonspecific marker of inflammation. Now accumulating epidemiological and clinical evidence showed that CRP was an independent risk factor for atherosclerotic vascular disease [Lagrand et al., 1999; Pepys and Hirschfield, 2003]. A large amount of published data indicated that CRP acted as a robust and independent risk marker in the prediction of primary and secondary adverse cardiovascular events [Devaraj et al., 2009]. In addition, in vitro experiments also showed that CRP could cause a number of biological changes in endothelial cells, smooth muscle cells, and macrophages which are considered to promote lesion progression, revealing that CRP appears to participate in the pathogenesis of atherosclerosis [Pasceri et al., 2000; Verma et al., 2006; Devaraj et al., 2009].

Oxidative stress may result from increased generation and/or inadequate removal of Reactive oxygen species (ROS). Excessive ROS generation causes the damage of proteins, lipids, and DNA [Perez-Matute et al., 2009]. Proteins, the main executor of cellular function, are one of the major targets of oxygen free radicals and other reactive species. Oxidation of proteins introduces carbonyl groups, which modulate biochemical characteristics of proteins such as enzymatic activity [Levine, 1983; Climent et al., 1989], DNA binding activity of transcription factors, and the susceptibility to proteolytic degradation [Rivett, 1986; Wolff and Dean, 1986]. Oxidative stress can injure the endothelium, leading to the dysfunction of endothelial cells and pathogenesis of atherosclerosis. Moreover, oxidative proteins such as oxidative low density lipoprotein (Ox-LDL) has been considered as an indicator of vascular disease because Ox-LDL could induce endothelial cell damage and dysfunction, stimulate the migration and proliferation of smooth muscle cells, mediate the formation of foam cells, which are the key events during pathogenesis of atherosclerosis.

NADPH oxidase (NOX) enzymes have been shown to be involved in cardiovascular physiology and pathophysiology [Barbieri et al., 2003; Abe et al., 2006]. It was also reported that NOX-mediated oxidative stress is a major mechanism for monocyte activation upon multiple stimuli [Yasunari et al., 2002]. Thus, we proposed that NOX-mediated oxidative stress might play a key role in CRP-induced monocyte-endothelial cell adhesion.

In the present study, we show novel data indicating that CRP could promote the adhesion of monocytes to endothelial cells via NOX-mediated oxidative stress. We also provide direct evidence for the proinflammatory effect of CRP on human monocytes by up-regulation of MCP-1 expression, which may facilitate the adhesion of monocytes to endothelial cells.

MATERIALS AND METHODS

PATIENTS

We collected 36 patients with carotid IMT incrassation (IMT$\geq$0.9 mm) for this study, according to European Society of Hypertension-European Society of Cardiology Guidelines Committee. All patients were free from clinical and instrumental indications of diabetic mellitus and severe hepatic and renal dysfunction. Thirty-four healthy control (HC) volunteers, matched for age, sex, and lifestyle, were recruited from the local community. All participants underwent physical examination and completed questionnaire on medical history, family diabetic history, and smoking habit. The local Ethical Committee had approved the study protocol. All subjects gave written informed consent to participate in this study.

ANALYSIS OF CLINICAL INDICATIONS

Plasma glucose level was examined with glucose oxidase method (Beckman). Total cholesterol (TC), triglycerides (TG), LDL-C, and high density lipoprotein (HDL)-C levels were measured using the kits from Sigma Diagnostics. Serum CRP and MCP-1 levels were determined with high sensitive microparticle enzyme immunoassay (Boster).

Serum malondialdehyde (MDA) level was measured using TBARS assay kit (Cell Biolabs). The carbonylation of total proteins in serum was determined using a commercial kit (Cayman Chemical) according to manufacturer's instructions.

MEASUREMENT OF CAROTID IMT

All studies were performed by an experienced vascular sonographer, using an Acuson Sequoia 512 mainframe (Acuson, Mountain View, California) with a 5 to13.0-MHz linear-array transducer. All studies followed a predetermined, standardized scanning protocol for the right and left carotid arteries. The proximal part of the carotid bulb was identified, and the segment of the common carotid artery 1–2 cm proximal to the bulb was scanned. The image was focused on the posterior wall, and the resolution box function was used to magnify the arterial far wall. Two angles were used in each case: Anterior oblique and lateral. All scans were digitally stored for subsequent off-line analysis. Two end-diastolic frames of the best image quality were selected and analyzed for maximum IMT, and the average reading from these two frames was calculated for both the right and left carotid arteries. Moreover, the maximum IMT of the carotid bulb regions was examined on both sides. The images were analyzed off-line, unaware of the individuals' clinical details.

ISOLATION OF HUMAN MONOCYTES

The human monocytes were isolated as described previously [Zhao et al., 2002; Zhao et al., 2003]. In brief, the cells were prepared from heparinized blood within 4 h after collection from donors. The mononuclear cells were obtained by centrifugation over a Ficoll-Paque density solution. The platelets were removed by several washes through serum. Mononuclear cells were incubated in serum-coated cell culture flasks for 3 h. After washing away nonadherent cells, the adherent cells were released with 5 mmol/L EDTA. The purity was evaluated by nuclear and CD14 staining, the viability was evaluated by Trypan blue exclusion. The monocyte suspensions were used only if purity was >90% and viability was >95%.

ISOLATION AND CULTURE OF ENDOTHELIAL CELLS

Human umbilical vein endothelial cells (HUVECs) were harvested enzymatically from infant umbilical cords under sterile conditions and established as primary cell cultures in phenol red-free M199

(Gibco Life Sciences) with 20% serum. The endothelial cells were cultured with 90% confluence in 24-well plate coated with gelatin. The purity was evaluated by factor VIII related antigen staining, and the viability was evaluated by Trypan blue exclusion.

MONOCYTE-ENDOTHELIAL CELL ADHESION ASSAY

The isolated human monocytes were cultured in RPMI1640 with 10% bovine serum at 37°C in 10% CO_2 for 2 h before treatment. The endothelial cells were pretreated with CRP for 8 h before the adhesion assay. The monocytes were treated (1) with 10 μg/ml CRP for 4 h, (2) with 5 μmol/L diphenyliodinium (DPI) for 30 min before 10 ug/ml CRP treatment for 4 h, (3) with 100 μmol/L apocynin for 30 min before 10 μg/ml CRP treatment for 4 h. After the treatment, 7×10^5 monocytes in 500 μl RPMI1640 were added to the endothelial monolayer cultured in 500 μl M199 and co-incubated for 1 h in a mixture of RPMI1640 and M199 at 37°C under 5% CO_2.

To calculate the adhesive rate, nonadherent cells were removed and collected by gentle washing by pipetting. The suspensions were used for counting cell numbers with a Neubauer hemocytometer (Weber Scientific). The adhesive rate = (Number of total monocytes)−(Number of nonadherent monocytes)/Number of total monocytes.

Monocyte-endothelial cell adhesion assay was performed in monocytes isolated from five healthy donors.

MEASUREMENT OF ROS GENERATION IN MONOCYTES

Monocytes (3×10^5 cells/ml) were incubated with various pharmacological reagents such as 5 μmol/L DPI, 100 μmol/L apocynin, 2 μmol/L Hypericin and 1 μmol/L phorbol 12-myristate13-acetate (PMA) (Sigma) for 30 min, followed by incubation with 10 μg/ml CRP for 4 h at 37°C. Finally ROS generation of the cells was detected using a commercial kit as described previously [Yuan et al., 2010]. Briefly, cells were incubated with DCF-DA (5 μmol/L) for 40 min at 37°C. After washing three times with Hanks' solution, the 2′7′-dichlorofluorescein fluorescence was measured by FACS (BD VantageSE) with an excitation/emission wavelength of 488/525 nm. Separate experiments were performed in monocytes isolated from five healthy donors.

RNA EXTRACTION AND REVERSE TRANSCRIPTION

The RNA from monocytes was extracted using Trizol Regent following the instruction. Reverse transcription of RNA was performed with A3500 kit (promega) according to the manufacturer's instruction.

QUANTITATIVE REAL-TIME PCR

Real-time PCR was performed using A7500 Real-Time Thermal Cycler (ABI). The following primers were used for real-time PCR: gp91phoxforward: CAAGATGCGTGGAAACTACC, reverse: TTGAGAATGGATGCGAAGG; p22phox forward: ATTGTGGCGG GCGTGTT, reverse: CGGCGGTCATGTACTTCTGTC; p47phox forward: CAGTCATGGGGGACACCTT, reverse: GACAGGTCCT GCCATTTCAC, MCP-1 forward: GCCTCCAGCAT GAAAGTCTC, reverse: AGGTGACTGGGGCATTGAT, GAPDH forward: TGCACCACCAACTGCTTAGC, reverse: GGCATGGACTGTGG TCATGAG. Amplification was carried out as recommended by the manufacturer (ABI). Separate experiments were performed in monocytes isolated from five healthy donors.

WESTERN BLOT

The monocytes were homogenized in lysis buffer (5 mmol/L EDTA, 150 mmol/L KCl, 0.5% NP-40, and 10 mmol/L Tris-HCl, pH 7.4) containing a protease inhibitor cocktail complete (Sigma). Cell lysates (10–30 μg protein) were separated by 10% SDS–PAGE, transferred to PVDF membrane (Millipore), blocked with 5% nonfat dry milk for 60 min, and probed with the antibodies at 4°C overnight. The blots were incubated with HRP-conjugated anti-IgG, followed by detection with ECL (Santa Cruz). The antibodies against p38MAPK, phosphor-p38MAPK (Thr180/Tyr182), and β-actin were purchased from Cell Signaling Technology. Separate experiments were performed in monocytes isolated from five healthy donors.

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1×10^4 monocytes were harvested and suspended with 120 μl PBS, adhered to coverslips by centrifugal slide stainer, fixed for 10 min in pre-cold methanol in −20°C, followed by incubation with rabbit anti-p65, p47phox or CD14 antibodies at 37°C for 60 min. The cells were labeled with TRITC-conjugated anti-rabbit IgG at 37°C for 60 min and incubated with DAPI at room temperature for 5 min. Finally, the coverslips were mounted with DABCO. Images were visualized with confocal microscopy.

STATISTICAL ANALYSIS

Data are expressed as means ± SD. Differences in the demographic and clinical characteristics among subgroups of subjects were assessed by one-way ANOVA and a Scheffe' post hoc test was used to examine differences between groups when significance was achieved, otherwise, a Kruskal–Wallis followed by a Mann–Whitney U-test was utilized. Pearson's correlation test was used to establish the correlation between continuously distributed variables. Statistical significance was established as $P < 0.05$. All analysis was performed by SPSS software (version 11.5).

RESULTS

PATIENTS WITH CAROTID IMT INCRASSATION DISPLAYED HIGHER TG AND LOWER HDL-C LEVEL

The subjects involved in this study were divided into carotid IMT incrassation (IP) group and HC group according to the IMT measurement by sonographer (Fig. 1A -a).

The demographic and clinical characteristics of subjects were summarized in Table I. No significant difference in age and sex distribution, the levels of glucose, LDL-C and TC were found between patients with IP and HC group. As expected, patients with carotid IMT incrassation displayed significantly higher TG level and lower HDL-C level compared with the HC group.

CAROTID IMT WAS ASSOCIATED WITH ABNORMAL LIPID METABOLISM AND INFLAMMATION IN PATIENTS WITH CAROTID IMT INCRASSATION

As shown in Figure 1A,B,D, compared with the HCs, patients with carotid IMT incrassation displayed significantly increased levels of carotid IMT(1.16 ± 0.25 mm vs. 0.70 ± 0.10 mm, $P < 0.01$), Hs-CRP(3.02 ± 3.31 ug/ml vs. 0.24 ± 0.54 ug/ml, $P < 0.01$) and

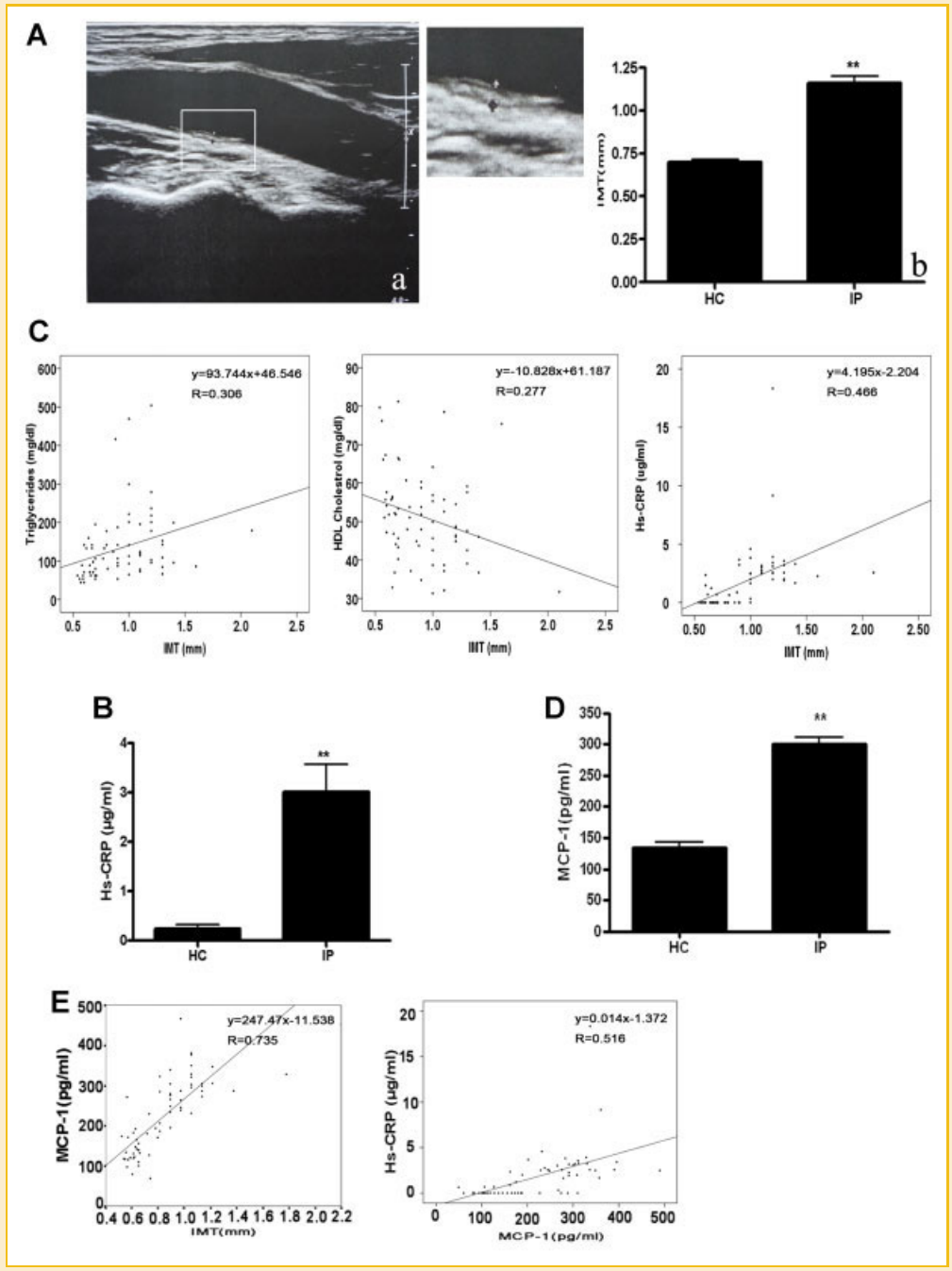

Fig. 1. Carotid IMT was associated with abnormal lipid metabolism and inflammation in patients with carotid IMT incrassation. The representative figure of IMT sonography of subject with IMT incrassation was shown. The arrow indicated the IMT, and the area in the frame was magnified and lay by side (A-a). Compared with the healthy controls ($n = 34$), patients with carotid IMT incrassation ($n = 36$) displayed significantly increased levels of carotid IMT (A-b), Hs-CRP (B), MCP-1 (D). A significant positive bivariate correlation between carotid IMT and TG ($P < 0.01$) and Hs-CRP ($P < 0.01$), while a significant inverse correlation between carotid IMT and HDL-C ($P < 0.05$), was found in all tested subjects (C). Moreover, there is a significant positive bivariate correlation between MCP-1 and carotid IMT ($P < 0.01$) and Hs-CRP ($P < 0.01$) (E). $^{*}P < 0.05$, $^{**}P < 0.01$.

MCP-1(300.06 ± 91.92 pg/ml vs. 136.96 ± 56.21pg/ml, $P < 0.01$). We next analyzed the relationship between carotid IMT and clinical characteristics. A significant positive bivariate correlation was found between carotid IMT and TG ($P < 0.01$), and Hs-CRP ($P < 0.01$) in all tested subjects (Fig. 1C). And there was a significant inverse correlation between carotid IMT and HDL-C ($P < 0.05$).

MCP-1 are the major regulators of monocyte trafficking. Previous study has shown that MCP-1 deficiency abolished the adhesion and infiltration of monocytes, and delayed progression to atherosclerosis [Kuziel et al., 1997]. In the present study, we found a significant positive bivariate correlation between MCP-1 and carotid IMT ($P < 0.01$) and Hs-CRP ($P < 0.01$) (Fig. 1E). These results indicated

TABLE I. Demographical and Clinical Characteristics of the Subjects

	HC (IMT < 0.9 mm)	IP (IMT≥0.9 mm)	*P*
Number	34	36	1
Age (years)	51 ± 4	53 ± 9	0.103
FPG (mg/dl)	97 ± 6	96 ± 18	0.707
Total cholesterol (mg/dl)	203 ± 41	192 ± 44	0.294
Triglycerides (mg/dl)	104 ± 67	165 ± 103	0.004*
HDL cholesterol (mg/dl)	54 ± 11	48 ± 11	0.043*
LDL cholesterol (mg/dl)	119 ± 39	117 ± 41	0.617
Body mass index (kg/m^2)	23 ± 3	24 ± 2	0.125
Systolic blood pressure (mm Hg)	118 ± 11	122 ± 14	0.543
Diastolic blood pressure (mm Hg)	76 ± 7	77 ± 9	0.104

Data are means ± SE.
*$P < 0.05$ compared with control group.

that carotid IMT was associated with abnormal lipid metabolism and inflammation in patients with carotid IMT incrassation.

OXIDATIVE STRESS OCCURRED IN PATIENTS WITH CAROTID IMT INCRASSATION

To investigate whether oxidative stress occurs in patients with carotid IMT incrassation, we measured the lipid peroxidation (MDA production) and protein carbonylation in serum. The MDA level was higher in patients with carotid IMT incrassation (6.91 ± 1.05 μmol/L) than that in the HCs (4.95 ± 2.63 μmol/L) ($P < 0.01$) (Fig. 2A). We also found that the carbonylation levels of serum proteins were enhanced in patients with carotid IMT incrassation (1.69 ± 0.39) compared with the control group (1.22 ± 0.64) ($P < 0.01$) (Fig. 2C). Moreover, a significant positive bivariate correlation was revealed between MDA and TG level ($P < 0.05$), and Hs-CRP ($P < 0.01$), and MCP-1 ($P < 0.01$), whereas a significant inverse correlation occurred between MDA and HDL-C ($P < 0.05$) (Fig. 2B).

It has been suggested that members of NOX family, including NOX1, NOX2, NOX3, NOX4, and NOX5, are main enzymatic sources of ROS in different tissues and cells. Using RT-PCR, we have found the expression of NOX2 (gp91phox) but not NOX1, NOX3, NOX4, and NOX5 in monocytes (data not shown). Moreover, gp91phox and subunit p22phox, but not p47phox, was up-regulated in the monocytes in patients with carotid IMT incrassation as shown by real-time PCR (Fig. 2E).

P47phox is considered as a subunit chiefly responsible for NOX activation. In the resting cells, p47phox exists in the cytoplasm, while under oxidative stress condition it translocates to the cell membrane, where it associates with cytochrome b558 to assemble the active oxidase. We therefore investigated subcellular localization of p47phox by confocal immunofluorescence microscopy. As shown in Figure 2F, p47phox had a cytoplasmic distribution in the monocytes in controls, but showed a cell membrane distribution in the monocytes of patients, suggesting increased NOX activity in the monocytes in patients with carotid IMT incrassation.

We further analyzed the relationship between gp91phox expression in monocytes and levels of TG, Hs-CRP, and MCP-1 in serum. As shown in Figure 2G, a significant positive bivariate correlation appeared between gp91phox and TG ($P < 0.01$), Hs-CRP ($P < .0.01$), and MCP-1 levels ($P < 0.01$), raising the possibility that increased levels of CRP and MCP-1 were associated with NOX2-mediated oxidative stress in patients with carotid IMT incrassation.

CRP PROMOTED MONOCYTE-ENDOTHELIAL CELL ADHESION, ACCOMPANIED BY UP-REGULATION OF MCP-1 EXPRESSION

To investigate the role of CRP in monocyte-endothelial cell adhesion, we preformed the monocyte-endothelial cell adhesion

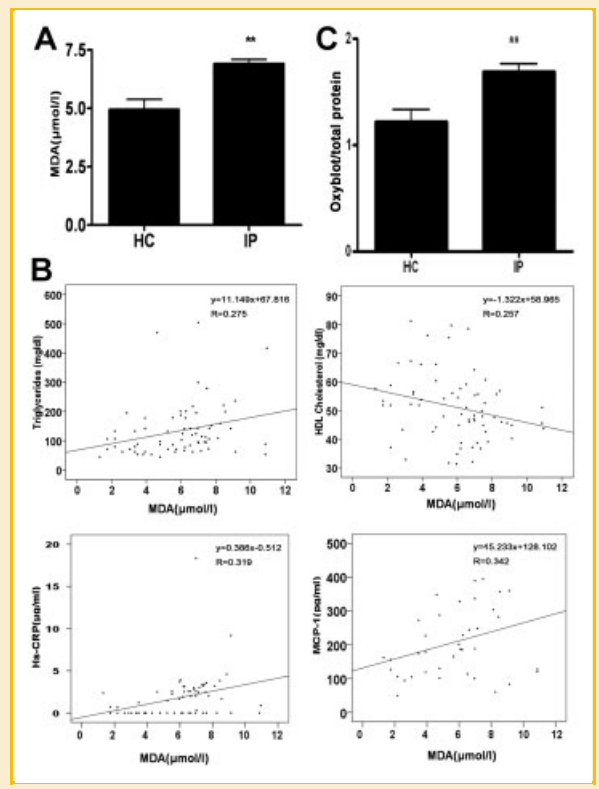

Fig. 2. Oxidative stress occurred in patients with carotid IMT incrassation. Serum MDA and protein carbonylation levels were enhanced in patients with carotid IMT incrassation compared with the control group (A, C). A significant positive bivariate correlation was revealed between MDA and TG level ($P < 0.05$), and Hs-CRP ($P < 0.01$), and MCP-1 ($P < 0.01$), whereas a significant inverse correlation occurred between MDA and HDL-C ($P < 0.01$) (B). The picture of CD14 staining in the isolated cells, showing >90% cells were CD14 positive cells, Bar = 10 μm (D). Gp91phox and subunit p22phox, but not p47phox, was up-regulated in the monocytes in patients with carotid IMT incrassation as shown by real-time PCR (E). P47phox had a cytoplasmic distribution in the monocytes of the controls, but showed a cell membrane distribution in the monocytes of patients. Bar = 10 μm (F). Moreover, a significant positive bivariate correlation appeared between gp91phox and TG ($P < 0.01$), Hs-CRP ($P < .0.01$), and MCP-1 levels ($P < 0.01$) (G).*$P < 0.05$, **$P < 0.01$.

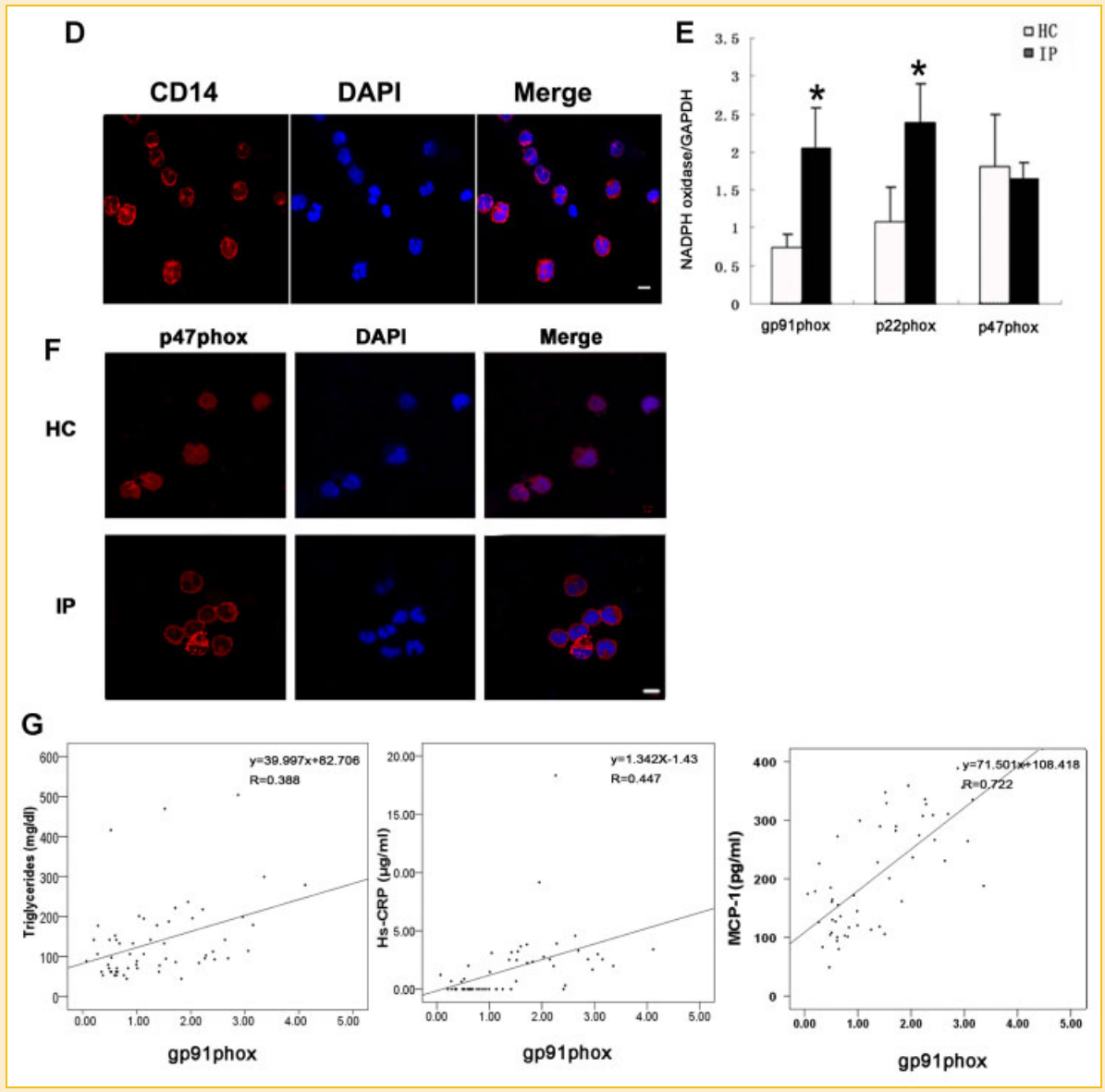

Fig. 2. (*Continued*)

assay. As indicated by adhesion rate, recombinant human CRP at high concentration (10 μg/ml) promoted monocyte-endothelial cell adhesion ($22.98 \pm 1.09\%$ vs. $28.54 \pm 1.05\%$, $n = 5$, $P < 0.05$) (Fig. 3A), accompanied by up-regulation of MCP-1 expression (1.00 ± 0.07 vs. 2.38 ± 0.30, $n = 5$, $P < 0.05$) (Fig. 3B).

CRP STIMULATED GENERATION OF ROS IN MONOCYTES BY ACTIVATION AND UP-REGULATION OF NOX

Based on our data, we hypothesized that CRP might stimulate the generation of ROS in the monocytes through activation and up-regulation of NOX. To test this hypothesis, different concentrations of CRP were added into suspension of monocytes. Compared with the control, CRP significantly increased ROS generation in the monocytes in dose-dependent manner, as indicated by flow cytometry (Fig. 4A). However, CRP-stimulated ROS generation was inhibited by DPI and apocynin, the inhibitors of NOX, indicating that NOX could be a leading candidate for production of ROS in the monocytes. We also found that PMA, an activator of protein kinase C (PKC), markedly elevated the CRP-stimulated ROS generation, while hypericin, an inhibitor of PKC, abolished this effect (Fig. 4B). Moreover, CRP enhanced the expression of gp91phox and p22phox, but not p47phox, in the monocytes (Fig. 4C). Taken together, the results demonstrated that CRP stimulated generation of ROS in the monocytes by activation and up-regulation of NOX.

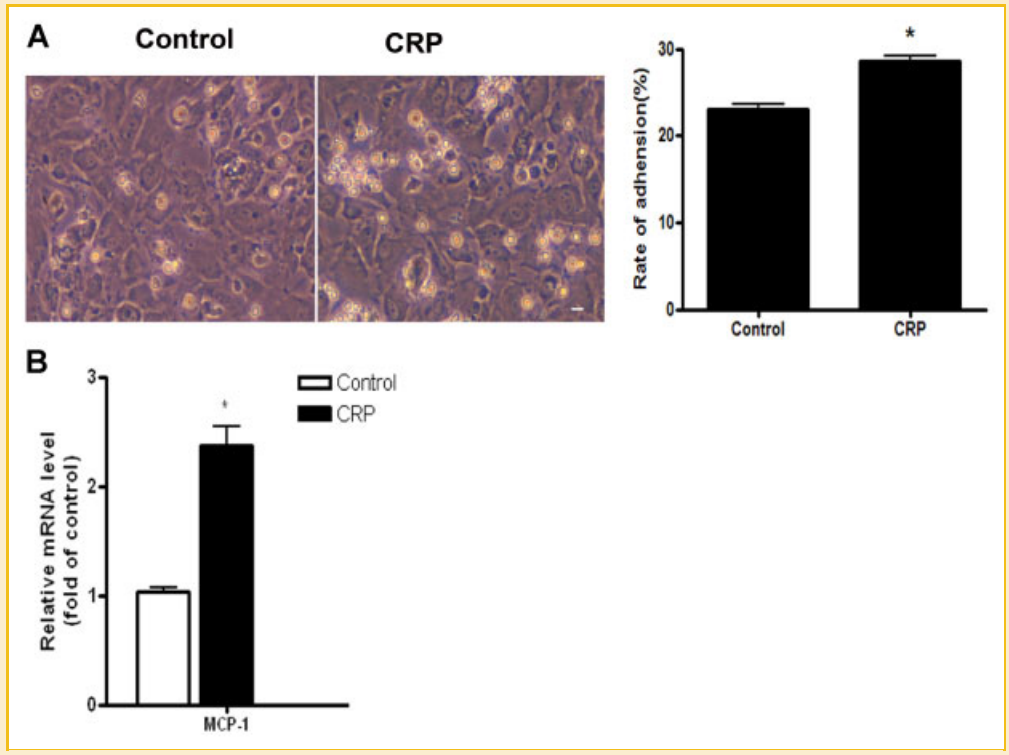

Fig. 3. CRP promoted monocyte-endothelial cell adhesion, accompanied by up-regulation of MCP-1 expression. Recombinant human CRP at high concentration (10 μg/ml) promoted monocyte-endothelial cell adhesion, Bar = 20 μm (A), accompanied by up-regulation of MCP-1 expression in the monocytes isolated from five volunteers (B). $^{*}P < 0.05$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

NOX INHIBITOR REVERSED CRP-INDUCED MONOCYTE-ENDOTHELIAL CELL ADHESION

To further assess the role of NOX in CRP-induced monocyte-endothelial cell adhesion, the monocytes were treated with NOX inhibitor DPI or apocynin for 30 min, followed by incubation with 10 μg/ml CRP for 4 h at 37°C. The monocyte-endothelial cell adhesion assay showed that DPI or apocynin suppressed CRP-induced monocyte-endothelial cell adhesion (Fig. 5A).

Most importantly, DPI or apocynin also inhibited CRP-stimulated up-regulation of MCP-1 expression in the monocytes (Fig. 5B). It is considered that NFκ B and p38 MAPK signaling pathways are involved in MCP-1 expression [Takahashi et al., 2008]. Thus, we analyzed the effect of CRP on activation of NFκ B and p38 MAPK signaling pathways, and investigated whether apocynin abolished this effect. As shown in Figure 5C,D, CRP led to translocation of p47phox and activation of NFκB, as assessed by translocation of p65, one member of NFκB family which binds p50 or p52 to form a dimeric complex of NFκB, into the nucleus. However, apocynin partially blocked these translocations induced by CRP treatment. Moreover, DPI and apocynin also rescued CRP-induced phosphorylation of p38MAPK (Fig. 5E).

DISCUSSIONS

Investigation of the role of CRP in atherosclerosis mainly focuses on vascular endothelial cells and smooth muscle cells, which may reflect the commonly accepted hypothesis of endothelial dysfunction as the key step to atherogenesis. We hypothesized that systemically circulating monocytes may also play an active role in atherogenesis in parallel with endothelial dysfunction. In the present study, we provide novel experimental evidences showing the important role of NOX in CRP-induced monocyte-endothelial cell adhesion in patients with carotid IMT incrassation. CRP stimulates the generation of ROS in the monocytes by activation and up-regulation of NOX, leading to increased expression of MCP-1, in turn promotes monocyte-endothelial cell adhesion.

Carotid IMT incrassation represents a pathophysiological status of the vascular wall and is a useful biomarker for atherosclerotic vascular disease. It has been shown that elevated carotid IMT was associated with hypertension [Takiuchi et al., 2004], androgenetic alopecia [Dogramaci et al., 2009] and inflammation [Baldassarre et al., 2008]. As shown in Figure 1C, we found a significant positive bivariate correlation between carotid IMT and serum CRP concentration, suggesting that CRP might be a new risk factor for carotid IMT incrassation. Similarly, a significant positive bivariate correlation was found between carotid IMT and serum MCP-1 level. These results raised a possibility that CRP might act as a proinflammatory factor to induce MCP-1 expression which promoted monocyte-endothelial cell adhesion. Recent studies have shown that CRP could play crucial role in monocyte-endothelial cell adhesion by inducing the expression of adhesion molecules and chemokines of endothelial cells [Pasceri et al., 2000; Pasceri et al., 2001]. It is reported that CRP could induce MCP-1

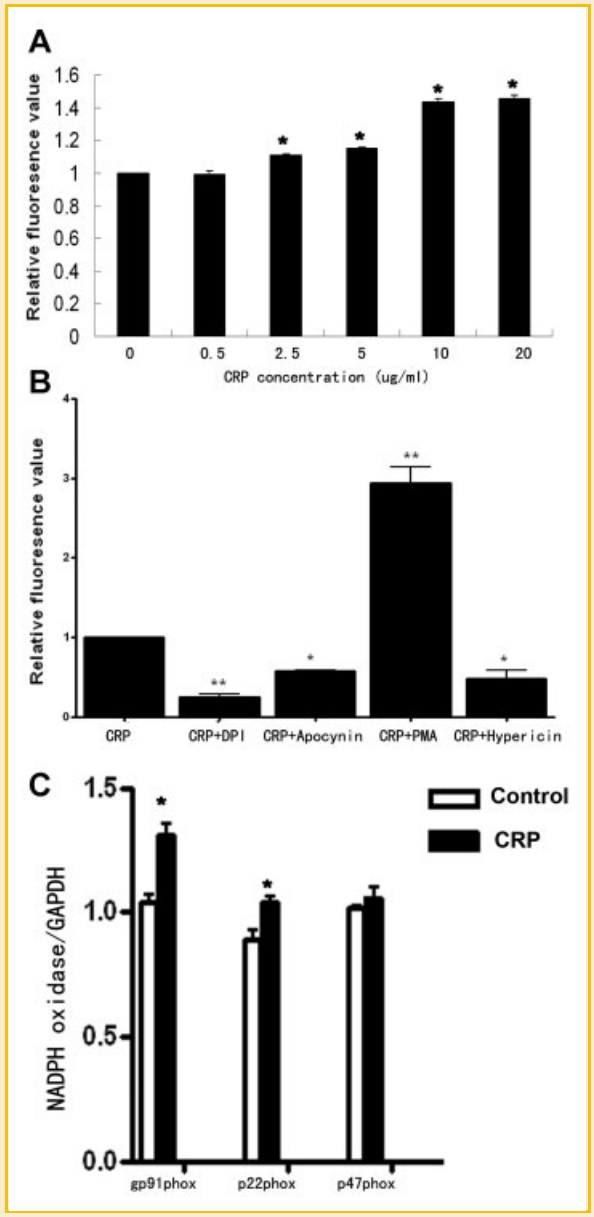

Fig. 4. CRP stimulated generation of ROS in the monocytes by activation and up-regulation of NADPH oxidase. Different concentrations of CRP were added into the suspension of monocytes isolated from five volunteers. Compared with control, CRP significantly increased ROS generation in the monocytes in dose-dependent manner, as indicated by flow cytometry (A). CRP-stimulated ROS generation was inhibited by DPI and apocynin, the inhibitors of NADPH oxidase. PMA, an activator of protein kinase C (PKC), markedly elevated the CRP-stimulated ROS generation, while hypericin, an inhibitor of PKC, abolished this effect (B). Moreover, CRP enhanced the expression of gp91phox and p22phox, but not p47phox, in the monocytes (C). $^{*}P < 0.05$.

expression in the endothelial cells, which acted as the major ligand for CCR2 and played a key role in monocyte infiltration. Disruption the interaction between MCP-1 and CCR2 caused severe decline in the leukocyte adhesion and monocyte infiltration in mice, indicating the importance of MCP-1 in this process [Kuziel et al., 1997].

However, little attention has been played on the effect of CRP on monocytes. Here, we found that the levels of serum CRP and MCP-1 were coordinately increased in patients with carotid IMT incrassation as indicated by Figure 1B,D. Moreover, incubation of the monocytes with 10 μg/ml CRP induced increased expression of MCP-1 in the monocytes (Fig. 3B). Thus, it was emphasized that the monocytes might also participate in monocyte-endothelial cell interaction by MCP-1 expression. Elevated MCP-1 levels in serum are secreted not only from the endothelial cells but also from the monocytes.

Oxidative stress is now recognized for its critical role in the pathogenesis of atherosclerosis [Furukawa et al., 2004]. It was originally accepted that the ROS generated from infiltrated monocytes contributes to atherosclerotic lesion formation. Our results demonstrated the oxidative stress state is associated with multiple factors including increased levels of TG, CRP, MCP-1 and decreased HDL level in patients with carotid IMT incrassation as indicated by Figure 2. Thus, the oxidative stress state of the monocytes may be a consequence of several risk factors. It is difficult to determine which one is the main factor involved in oxidative stress of the monocytes in patients with carotid IMT incrassation. Therefore, we sought to extend these observations to the cellular model in which 10 μg/ml CRP was added into the monocytes isolated from five volunteers. As shown in Figure 4, the results indicated that CRP significantly increased ROS generation in the monocytes.

It has been suggested that NOX is main enzymatic source of ROS in phagocytes and vascular cells [Cathcart, 2004; Takeya et al., 2006]. Our results demonstrated that gp91phox and subunit p22phox, but not p47phox, was up-regulated in the monocytes in patients with carotid IMT incrassation. Moreover, a significant positive bivariate correlation appeared between gp91phox and TG, CRP and MCP-1 levels in patients with carotid IMT incrassation, suggesting that increased levels of CRP and MCP-1 were associated with NOX-mediated oxidative stress in patients with carotid IMT incrassation (Fig. 2). In vitro study, we also found that CRP enhanced the expression of gp91phox and p22phox, but not p47phox, in the monocytes(Fig. 4C).

Most importantly, DPI or apocynin, the inhibitors of NOX, could abolish CRP-induced increased ROS generation and expression of MCP-1 in monocytes. DPI or apocynin could also suppress CRP-stimulated monocyte-endothelial cell adhesion.

In addition, it is known that NOX is activated by different manners on different stimuli [Sun et al., 2002; Barbieri et al., 2003]. A powerful step in NOX activation is phosphorylation and translocation of p47phox. As indicated by Figure 2E, we found that p47phox was translocated to membrane in the monocytes in patients with carotid IMT incrassation, while it distributed in cytoplasm in the controls. In vitro experiments also showed that CRP could stimulate the translocation of p47phox and ROS generation, suggesting an important role of p47phox translocation in CRP-induced NOX activation (Fig. 5). Moreover, we also separated the plasma membrane and cytosol from the monocytes to investigate the localization of p47phox by Western blot (data not shown). The result is consistence with immunofluoresence.

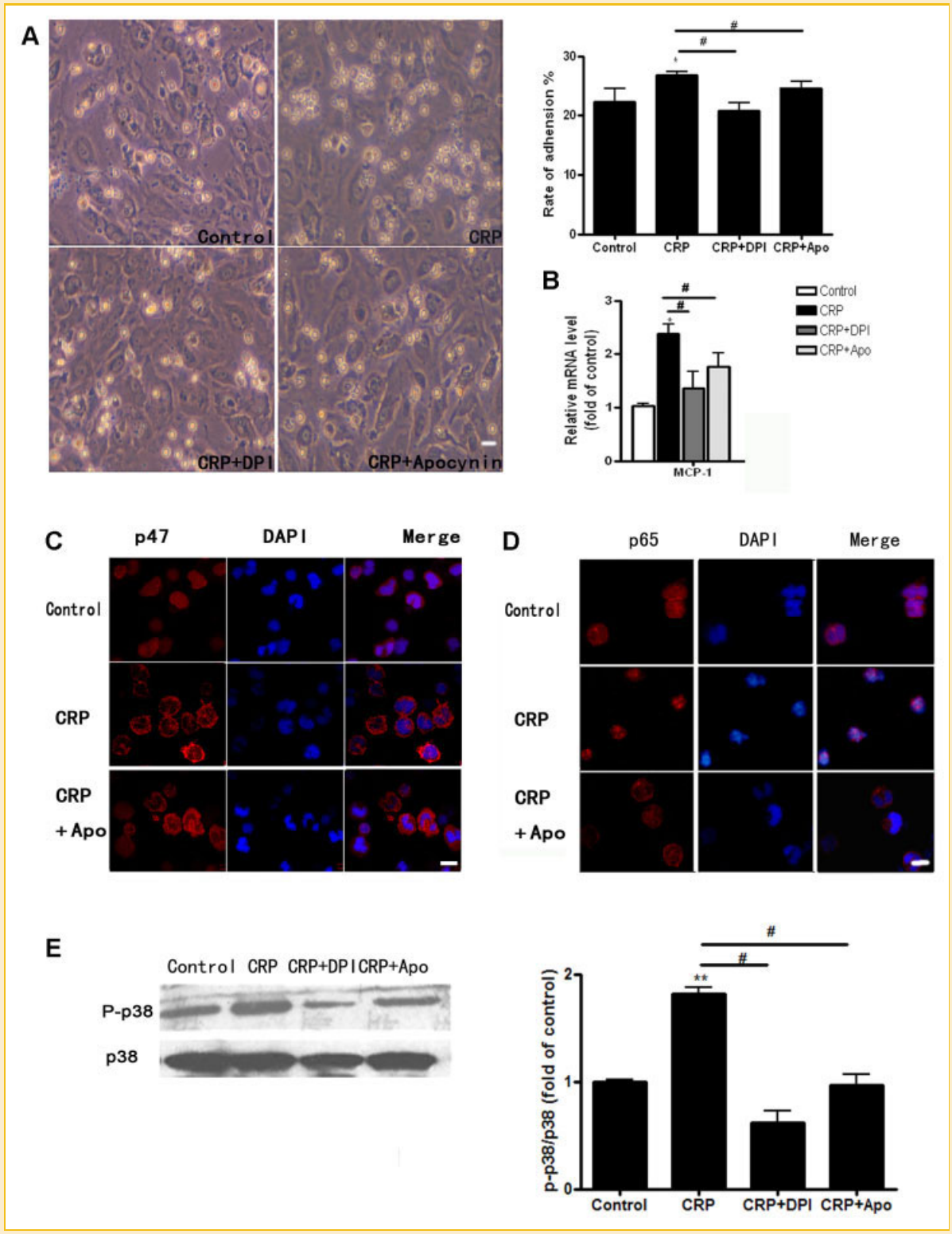

Fig. 5. NADPH oxidase inhibitor reversed CRP-induced monocyte-endothelial cell adhesion. The monocytes were treated with NADPH oxidase inhibitor DPI and apocynin for 30 min, followed by incubation with 10 μg/ml CRP for 4h at 37°C. Monocyte-endothelial cell adhesion assay showed that DPI and apocynin suppressed CRP-induced monocyte-endothelial cell adhesion Bar = 20 μm (A). DPI and apocynin also inhibited CRP-stimulated up-regulation of MCP-1 expression in the monocytes (B). Moreover, apocynin reversed CRP-stimulated translocation of p47phox Bar = 10 μm (C) and activation of NFκB as assessed by translocation of NFκB into the nucleus Bar = 10 μm (D). DPI and apocynin also rescued CRP-induced phosphorylation of p38MAPK as indicated by Western blot (E). $^{*}P < 0.05$ compared with control, $\#P < 0.05$ compared with CRP-treated group.

Kitada, M. suggested that the translocation of glomerular p47phox was required for oxidative stress in diabetic nephropathy [Barbieri et al., 2003; Piconi et al., 2008]. Moreover, it is reported that PKC regulates the activation NOX [Yamamori et al., 2000]. In the present study, we found that PMA, an activator of PKC, markedly elevated the CRP-stimulated ROS generation, while hypericin, an inhibitor of PKC, abolished this effect as indicated by Figure 4.

Oxidative stress leads to increased expression of proinflammatory cytokines and adhesion molecules by activation of redox-sensitive transcription nuclear factor NF-κB. Inactive NF-κB is restricted to the cytoplasm, while active NF-κB is translocated to the nucleus. In addition, it has been shown that ROS can trigger the activation of stress-sensitive serine/threonine kinase signaling pathways such as p38MAPK. Moreover, it is reported that NFκ B and p38 MAPK signaling pathways are involved in MCP-1 expression [Yasunari et al., 2002]. Our results showed that CRP led to activation of NFκB, as assessed by translocation of NFκB into the nucleus. However, apocynin partially blocked this translocation induced by CRP treatment. Moreover, apocynin also rescued CRP-induced phosphorylation of p38MAPK (Fig. 5).

Our data suggest that CRP not only promoted endothelium-monocyte adhension by the proinflammatory effect of CRP on human endothelial cells but also induced the proinflammatory state of the monocytes by inducing the expression of MCP-1 and activation of NFkB signaling. The activation of monocytes may play a direct proatherosclerotic role by mediating monocyte/lymphocyte trafficking and infiltration. Moreover, we show that NOX-mediated oxidative stress may be the underlying mechanism of CRP induced monocyte-endothelial cell adhesion, which reveals the targets of interferences and drug design for therapy.

Taken together, our results show that CRP promotes monocyte-endothelial cell adhesion accompanied by the elevation of MCP-1 and activation of NF-κB and p38 MAPK signaling pathways. This process is mediated by NOX-derived ROS production.

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