Interferon-γ and Lipopolysaccharide Potentiate Monocyte Tissue Factor Induction by C-Reactive Protein

Relationship With Age, Sex, and Hormone Replacement Treatment

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Background**—**Elevated plasma levels of C-reactive protein (CRP) in population studies and in patients with unstable coronary syndromes are predictive of future adverse events, including cardiac death and myocardial infarction, implicating inflammation in pathogenesis. Although CRP is considered a marker of inflammation, it induces monocyte tissue factor (TF) and may play a prothrombotic role in atherosclerosis and its complications.

Methods and Results**—**Peripheral blood mononuclear cells (PBMCs) from 79 healthy men and women aged 26 to 83 years and 21 healthy postmenopausal women taking hormone replacement therapy (HRT) were stimulated with CRP, lipopolysaccharide (LPS), interferon-γ (IFN), or their combination. Levels of CRP in the normal range (1 to 5 μg/mL) increased basal monocyte TF 4- to 6-fold and 40-fold at higher concentrations (25 μg/mL). Coincubation of LPS with CRP produced a greater-than-additive response. IFN did not induce TF but synergized with CRP to approximately double activity. There was a striking positive correlation between age and monocyte TF induction, with a dramatic rise on monocytes from postmenopausal women that was not apparent on cells from women taking HRT.

Conclusions**—**Synergy between CRP and inflammatory mediators may play a direct prothrombotic role in the pathogenesis of coronary atherosclerosis and its acute complications by increasing monocyte/macrophage TF. This may contribute to age and sex differences in coronary events and to the protective effects of HRT. **(*Circulation*. 2000;101:1785-1791.)**

Key Words: inflammation ■ coagulation ■ immune system ■ atherosclerosis ■ coronary disease

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C-reactive protein (CRP), an acute-phase reactant present in normal plasma at low levels, can increase >100-fold in response to inflammatory stimuli.[1–5] Plasma CRP levels at the upper end of the reference range in apparently healthy men and women are associated with increased risk of future cardiovascular events, independent of established lipid and nonlipid coronary risk factors.[3–8] In patients with unstable coronary syndromes, elevated CRP levels are associated with increased risk of death or infarction.[9]

CRP is secreted by hepatocytes in response to interleukin (IL)-6, a cytokine associated with raised CRP levels in unstable angina.[10] CRP accumulates in macrophage-rich regions in developing atherosclerotic lesions[11] and upregulates some macrophage proinflammatory cytokines.[12,13] CRP also induces monocyte tissue factor (TF),[14,15] but it is uncertain whether an elevated CRP level is simply a marker of ongoing inflammation to oxidized lipids or unidentified environmental agents or whether CRP has direct prothrombotic effects in atherosclerosis and its complications.[8,16]

TF, a potent activator of the extrinsic coagulation cascade, is considered to play an important role in atherosclerosis. It is expressed on macrophages in atherosclerotic plaque[17–20] and may contribute to acute thrombotic events associated with plaque rupture in unstable syndromes.[19,20] CD4$^+$ T lymphocytes occur in almost all stages of atherosclerosis and produce interferon (IFN), which may contribute to lesion formation.[21,22] IFN does not directly induce TF in blood monocytes[23,24] but synergizes strongly with lipopolysaccharide (LPS) to induce TF on inflammatory macrophages.[24]

Here, we show that IFN and LPS potentiate monocyte TF induction by CRP and report a remarkable relationship between TF induction and both sex and age, as well as a significant protective effect of hormone replacement therapy (HRT) in postmenopausal women. This may represent an important amplification mechanism for triggering coagulation in inflamed plaques that underlie unstable coronary syndromes.

Methods

Reagents

Media prepared with pyrogen-free distilled water (Travenol Labs) was filtered through Zetapore membranes (0.2 m; AMF, Cuno Inc) into heated glassware (3 hours at 250°C). Cycloheximide and actinomycin D were from Calbiochem. Human IFN-γ was from

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Bender & Co. LPS (*Escherichia coli* 055: B5) was from Difco; human CRP (>90% pure) and Hanks' balanced salt solution (HBSS) were from Sigma; RPMI 1640 was from GIBCO; and human factors VII, X, and Xa, factor Xa chromogenic substrate, murine MAb against human TF (HTF-1), and Spectrozyme LAL (Limulus amebocyte lysate) were from American Diagnostica Inc. IgG_1 isotype control antibody was from ICN ImmunoBiologicals. Pyrosol LAL buffer was from Associates of Cape Cod, Inc, and endotoxin-tested Lymphoprep was from Nycomed Pharma AS. CRP (selected after pretesting from 3 different sources), cytokines and media tested for endotoxin by the Limulus assay[25] contained <5 pg of endotoxin per milliliter.

Subjects

Peripheral venous citrated blood (30 mL) was collected from 79 healthy subjects (42 men and 37 women aged 26 to 83 years) and 21 healthy women (aged 50 to 74 years) taking HRT. Subjects with histories of connective tissue disease, cancer, acute infection, ischemic heart disease, hypercholesterolemia, hypertension, or diabetes, those taking medication (particularly aspirin or lipid-lowering drugs), and ex-smokers or current smokers were excluded. Subjects were grouped according to age and sex: group 1 consisted of men <50 years of age; group 2, premenopausal women <50 years old; group 3, men >50 years old; and group 4, postmenopausal women >50 years old. Group 5 (postmenopausal women >50 years old taking HRT and attending a menopause clinic) included 9 women with prior hysterectomy and 3 with prior hysterectomy plus oophorectomy. Five took oral conjugated estrogen (Premarin, 1.25 mg/d), and 16 had a 50-mg estradiol subcutaneous implant. In 15 women, estrogen was unopposed; 6 took additional medroxyprogesterone (5 to 10 mg/d). In 11 women, testosterone undecanoate (40 mg/d orally in 3 and 100 mg subcutaneous implant in 8) was also prescribed to improve well-being and libido.[26,27] Duration of HRT was 6 to 48 months (mean 22±12 months). 17β-Estradiol levels ranged from 99 to 859 pmol/L (mean±SD 495±233).

Measurement of Procoagulant

PBMCs were obtained by gradient centrifugation on Lymphoprep as described previously.[25] In some experiments, we enriched monocytes ($\approx$95% pure by nonspecific esterase staining) by incubating PBMCs for 2 hours at 37°C on serum-coated wells and harvesting the lymphocytes. Monocyte numbers (5% to 10% of PBMCs) did not vary significantly between groups. PBMCs in serum-free RPMI were incubated at 37°C (5% CO_2 in air) in 96-well plates (Nunc; 5×10^5 cells/250 μL) with stimulants at the doses indicated. In agreement with a previous report for CRP,[15] activity induced by CRP±IFN was obvious after 4 hours, was optimal between 12 to 16 hours, and then declined. PBMCs were routinely cultured for 16 hours, washed twice with RPMI 1640 to remove lymphocytes, and tested after 3 cycles of freezing at −80°C and thawing at 37°C. The 1-stage plasma recalcification assay was performed as described previously,[25] and activity was

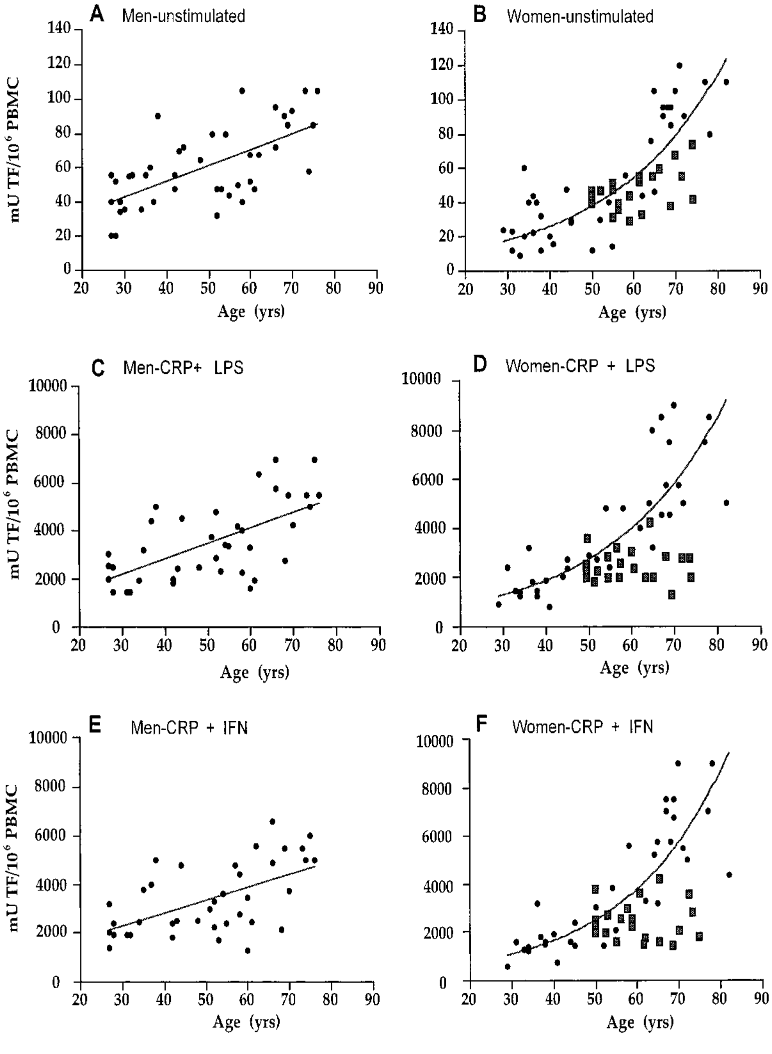

Figure 1. Induction of TF on monocytes from men (A, C, E) and women (B, D, F) of increasing age. Unstimulated PBMCs (A, B) or PBMCs stimulated with CRP (25 μg/mL) plus either LPS (10 pg/mL) (C, D) or IFN (200 U/mL) (E, F). Gray squares in B, D, and F represent responses of PMBCs from women receiving HRT. Values (mU TF/10^6 PBMC) represent mean responses (duplicate samples) in 1 subject, measured by plasma recalcification. Linear regression lines (for men) and exponential fits (for women not taking HRT) are superimposed on the data.

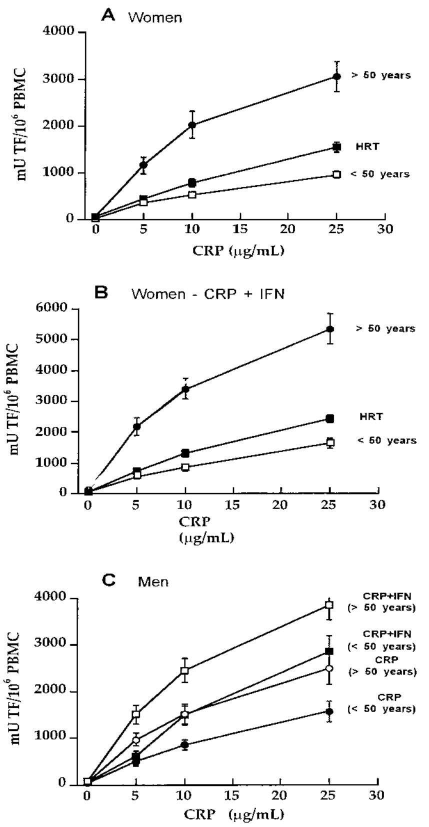

Figure 2. TF induction by CRP is dose dependent. Responses of PBMCs from women aged <50 years (□), >50 years (●), or >50 years taking HRT (■). CRP alone shown in A; CRP+IFN (200 U/mL) in B. C, TF induced by CRP on PBMCs from men (aged <50 years, ●; >50 years, ○) and by CRP+IFN (200 U/mL) (men aged <50 years, ■; >50 years, □). Each point represents mean±SEM of duplicate values of recalcification assay with PBMCs from 5 subjects per group.

expressed as milliunits (mU) of TF/10^6 cells by comparison with human brain powder as TF standard.[25]

Factor Xa generated after addition of factors VII and X was measured on viable cells with factor Xa–specific chromogenic substrate as described previously.[25] TF procoagulant was confirmed by incubation of PBMCs (10^6) with control IgG_1 or anti-TF MAb (20 μg/0.5 mL) for 1 hour on ice; factor VII was then added (50 μg/50 μL HBSS plus 3 mmol/L $CaCl_2$ plus 0.1% ovalbumin), and factor Xa generation was measured.

Statistical Analysis

Data are expressed as mean±SEM. Statistical analyses were performed with ANOVA, and the unpaired *t* test was used to compare TF levels on PBMCs between groups. Pearson correlation coefficient analysis was used to assess associations between values determined to be normally distributed. The relationship between age and procoagulant was analyzed by linear or nonlinear regression, and models were compared with r^2 and F values, inspection of residual plots, and an F ratio test (linear versus quadratic). Women taking HRT were significantly younger than women not taking HRT (mean age 57±7 versus 66±9 years, respectively; $P<0.01$), and the independent contributions of HRT and age were determined by stepwise multiple linear regression analysis. The influence of additional testosterone use was tested in a model that included age and HRT. Model R^2 was adjusted for sample size. A value of $P<0.05$ was considered statistically significant.

Results

CRP Synergizes With IFN to Induce TF

As little as 1 μg/mL CRP enhanced basal procoagulant activity (50±4 mU; n=29) ≈4-fold (233±44 mU), and basal procoagulant activity was enhanced 13-fold with 10 μg/mL CRP (670±130 mU), levels greater than those induced by 1 ng/mL LPS (440±58 mU). When LPS and CRP were combined, activity was somewhat greater than with each stimulant alone (Table 1).

IFN did not induce procoagulant activity and did not amplify the LPS-provoked response, but when combined with increasing concentrations of CRP, synergy was observed (Table 1 and Figure 2), particularly with ≥10 μg/mL of CRP ($P<0.001$ compared with unstimulated peripheral blood mononuclear cells [PBMCs]). Tumor necrosis factor provoked a weak response but did not synergize with CRP (not shown). Activity on monocytes depleted of lymphocytes and stimulated with CRP with or without LPS was only half that expressed by total PBMCs, and after CRP+IFN stimulation, it was only 25% of the unfractionated population.

TABLE 1. Age and Sex-Related Differences in TF Induction

	Age <50 y		Age >50 y	
	Men (G1) (n=20)	Women (G2) (n=17)	Men (G3) (n=22)	Women (G4) (n=20)
Control	50±4†	28±3	71±5†	75±7†§
LPS	246±57†	87±20	469±82†‡	690±113†§‖
CRP	1571±223*	965±78	2495±351†‡	3070±318†§‖
CRP+LPS	2761±378†	1764±187	4219±344†‡	5465±462†§‖
CRP+IFN	2854±342†	1620±163	3858±325†‡	5386±483†§‖

G1, G2, G3, and G4 indicate groups 1 through 4, respectively. For a definition of the groups, see Methods.

PBMCs stimulated with CRP (25 μg/mL), LPS (10 pg/mL), or IFN (200 U/mL) alone or in combination and recalcification times of lysed cells were determined. Data represent mean±SEM of mU TF/10^6 PMBC from the number of subjects (n) given.

All groups (G1–G4): $P<0.01$ for CRP+LPS or IFN vs CRP alone, CRP vs LPS, LPS vs control. For control and all stimulants: *$P<0.04$, †$P<0.01$ vs G2; ‡$P<0.05$, §$P<0.01$ vs G1; ‖$P<0.05$ vs G3.

TABLE 2. Comparison of TF Induction on Monocytes From Postmenopausal Women Taking and Not Taking HRT

	Unstimulated	LPS	CRP	CRP+LPS	CRP+IFN
Unadjusted for age					
Mean (SE), HRT (n=21)	54 (3)	403 (30)	1556 (106)	2339 (147)	2433 (126)
Mean (SE), no HRT (n=20)	75 (8)	690 (113)	3070 (318)	5465 (462)	5386 (483)
P	0.016	0.022	<0.001	<0.001	<0.001
Adjusted for age (multivariate)					
R^2 (age)	0.446	0.132	0.331	0.335	0.39
Coefficient	2	10	52	66	85
P	<0.001	0.19	0.01	0.03	0.006
Partial R^2 (HRT)	0.002	0.046	0.119	0.248	0.189
Coefficient	−3	−194	−1029	−2511	−2159
P	0.72	0.15	0.007	<0.001	<0.001
Model R^2 (age and HRT)	0.448	0.178	0.45	0.583	0.579
Adjusted model R^2 (age and HRT)	0.419	0.135	0.421	0.561	0.557
Estimate for testosterone use (multivariate model including age and HRT)					
Coefficient	15	136	142	484	382
P	0.11	0.41	0.75	0.46	0.61

Data in top 2 rows: mean (SEM) of TF (mU/10^6 PBMC) induced on unstimulated cells or PBMCs with LPS 10 pg/mL, CRP 25 μg/mL, IFN 200 U/mL, or their combination. *P* values for comparisons between postmenopausal women taking/not taking HRT are for unpaired *t* tests for data with unequal variance. Partial R^2, model R^2 (adjustment for small sample size), coefficients, and *P* values are for multiple linear regression with a model including age and HRT, as described in Methods. R^2 and *P* value for HRT represent increment in explained variance by adding HRT to a model already including age. Coefficients and *P* values for testosterone use are for a model including age and HRT. None of the estimates are significant.

Factor Xa generation was dependent on factors VII and X, and the magnitude of responses corresponded to those measured by plasma recalcification. Factor Xa generated by viable cells after stimulation with CRP more than doubled when coincubated with IFN (from 89±31 to 176±86 ng FXa/10^6 PBMC, n=6). Activity was inhibited by >90% with a neutralizing anti-TF monoclonal antibody (MAb), confirming the major contribution of surface-associated TF to the procoagulant response. TF antigen, localized by flow cytometry, was only expressed on monocytes ($CD14^+$), whereas lymphocytes ($CD14^-$) were negative (not shown).

The requirement for new RNA and protein synthesis for TF induction was confirmed by reduction of activity to basal levels when PBMCs were stimulated with CRP±LPS/IFN in the presence of 5 μg/mL cycloheximide or actinomycin D, respectively.

TF Induction Is Influenced by Sex and Age

Basal activity on monocytes from individuals >50 years old (Table 1, groups 3 and 4, and Figure 1, A and B) was low (71 to 75 mU) but was higher than on monocytes from young subjects; young women (group 2) expressed the lowest levels. Responses to 10 pg/mL LPS were significantly different between groups, and activity of cells from men <50 years old was about half that of men >50 years old (Table 1 and Figure 1C). Dramatic increases were observed with PBMCs from postmenopausal women (group 5): net stimulation by LPS was significantly greater (690±113 mU) than TF induced on cells from groups 1, 2, or 3, particularly from women <50 years old (87±20 mU). Figure 1A and 1B shows the rises in basal TF with increasing age, particularly on monocytes from women ($r=0.83$, $P<0.001$ for women; $r=0.62$, $P<0.001$ for men). Age-related responses to LPS ($r=0.44$, $P=0.007$ for men; $r=0.64$, $P<0.001$ for women; not shown) and CRP ($r=0.44$, $P<0.007$ for men; $r=0.76$, $P<0.001$ for women; not shown) followed a similar trend.

TF induced by CRP plus either LPS or IFN was also greatest on cells from older individuals (Table 1), particularly postmenopausal women (>5000 mU), and showed high correlation with increasing age ($r=0.83$ for CRP+LPS; $r=0.84$ for CRP+IFN; Figure 2, D and F). Six of 13 women >60 years old showed activity >7000 mU; responses of PBMCs from men in this age range never exceeded this level (Figure 1, C and E). Although significant, correlations between age and activity on monocytes from men ($r=0.54$ for CRP+LPS, Figure 1C; $r=0.50$ for CRP+IFN, Figure 1E) were consistently less than for women. There were no significant differences between linear and nonlinear fits in results from men and women, although in women, residual plots revealed signs of curvature consistent with a quadratic or exponential fit.

HRT Reduces Monocyte Responsiveness

Basal or induced TF on monocytes from women treated with estrogen did not differ significantly from that on monocytes from women also taking progestogen or additional testosterone, and therefore results in group 5 were pooled. No associations between reduced TF induction and duration of treatment (up to 4 years) or plasma levels of 17β-estradiol were obvious. Basal TF on monocytes from the HRT group was significantly less ($P=0.016$) than on cells from postmenopausal women, and activity induced by CRP (1556±106 mU) was approximately half that of untreated women (group 4, 3070±318 mU; Table 2). Multiple linear regression analysis confirmed that differences in mean ages did not account for the reduced TF in the HRT group. The partial R^2 for HRT in the CRP+LPS/IFN stimulation groups was of the same order of magnitude as for age, with $P<0.001$ and coefficients >2000 mU, which indicates a large effect of HRT after adjustment for age (Table 2). Interestingly, mean responses of PBMCs to CRP±LPS/IFN from women taking HRT approached levels in men aged <50 years (group 1, Table 1). Monocytes from 5 of 21 HRT subjects exhibited <2000 mU of TF (Figure 1, D and F), well within the range of activity induced by the combination stimulus on monocytes from women aged <50 years (Table 1). Testosterone use in women taking HRT did not influence these findings: when added to the model that included age and HRT, testosterone use was not an independent predictor of TF activity after any stimulation (all $P>0.4$), and a separate multivariate analysis that excluded women taking testosterone yielded almost identical results to the original analysis, with the same patterns of significance.

PBMCs from women aged >50 years were sensitive to as little as 5 μg/mL CRP (Figure 2A); TF activity induced by 10 μg/mL was some 3-fold more than that expressed by monocytes from younger women or those taking HRT. The trend was more obvious when increasing doses of CRP were combined with IFN (Figure 2B). PBMCs from men generated less obvious age-related responses with low concentrations of CRP (5 μg/mL), although coincubation of this concentration with IFN induced ≈3-fold more TF on cells from men aged >50 years. High CRP concentrations generated greater differences on monocytes from men of different ages (Figure 2C), although activity never reached the magnitude seen in women (Figure 2, A and B).

Discussion

This study provides new insights into mechanisms linking inflammation and coagulation, which may contribute to the progression and outcome of thrombotic events associated with atherosclerosis. Our findings are relevant to the strong predictive value of plasma CRP for cardiovascular risk and strengthen the hypothesis that CRP is associated with clinical events by altering clotting status[16] through induction of monocyte TF.

TF is proposed as a key mediator of thrombosis in atherosclerosis.[28,29] Circulating TF is detected in patients with unstable angina,[30,31] and high levels are expressed on macrophages in unstable plaques.[32,33] Here, we report responses of monocytes from healthy subjects to levels of CRP in the normal physiological range (1 μg/mL), with TF activity ≈4-fold above basal levels and even greater at higher concentrations (Figure 2). Considering the significantly enhanced responses in the elderly, particularly when LPS or IFN was added (Table 1, Figures 1 and 2), this mechanism may contribute to the increased risk of coronary events seen in population studies at plasma CRP concentrations >2 to 7 mg/L[6,34] and in studies of unstable coronary disease at levels >3 to 15.5 mg/L.[35,36]

Synergy with IFN is important because the development of atherosclerosis has a cell-mediated immune component[21] that includes IFN production by CD4$^+$ T lymphocytes, and because T-cell activation is present in unstable angina.[37] IFN doubled TF expression induced by CRP to 50 to 100 times basal levels (Table 1), and a critical role for lymphocytes for optimal responsiveness to CRP+IFN was indicated. Amplification of monocyte TF by contact with T lymphocytes is mediated, at least in part, by ligation of macrophage CD40 with CD40 ligand, a CD4$^+$ T-cell surface protein implicated in the pathogenesis of atherosclerosis and the triggering of acute coronary events.[38]

Coronary artery disease incidence increases with age and is greater in men than in age-matched women, although sex differences decline with age, and elevated CRP levels predict cardiac events in older men and women.[4–7,34] TF activity on monocytes from young women stimulated by CRP±LPS/IFN was significantly less than on cells from men of the same age (Table 1). In contrast, activity on monocytes from postmenopausal women, particularly those aged >60 years, increased exponentially to levels higher than those induced on monocytes from males of the same age (Figure 1). This mechanism may contribute in part to the age- and sex-related increases in risk and the predictive role of CRP. Postmenopausal women taking estrogen apparently have fewer cardiovascular events than untreated women,[39,40] although in the HERS trial (Heart and Estrogen/progestin Replacement Study),[41] HRT was not associated with reduced cardiovascular events; patients taking HRT had more events in the first year of treatment and fewer events in the fourth and fifth years, which indicates a biphasic response. Hypertension, insulin resistance, and hyperlipidemia are all beneficially affected by estrogen,[42] and estrogen retards plaque progression in animal studies.[43,44] Moreover, plasminogen activator inhibitor-1 levels are reduced in women taking oral HRT, which suggests an altered balance between coagulation and fibrinolysis.[45] Although the effects of HRT on plasma levels of components of coagulation do not indicate a protective effect,[42] our findings strongly support the view that HRT may be cardioprotective by affecting cellular procoagulants. TF activity on monocytes from women taking HRT was dramatically less than that of untreated women (Table 2), even with relatively low levels of CRP (Figure 2), and it approached activity exhibited by monocytes from young men (Table 1).

A previous study of LPS-stimulated monocytes from women taking estrogen indicated lower amounts of tumor necrosis factor, thromboxane B2, and TF than produced by cells taken before treatment; 12 months was required for stabilization of reduced responsiveness.[46] We failed to demonstrate an effect of estradiol in vitro on TF induced by CRP on monocytes from untreated women (not shown), which suggests an acquired response. Although mechanisms are unclear, they may exert their effects via an indirect pathway that regulates the synthetic capacity of monocytes, possibly through an effect on stem cells. If a period of some months is required to regulate monocyte responsiveness, this may explain the biphasic response in HERS.[41]

Results reported here suggest an important amplification loop linking some well-established risk factors for cardiovascular disease and inflammation. Deposits of CRP around collections of foam cells,[11] together with $CD4^+$ T cells producing IFN, could collectively induce high levels of TF, which might initiate thrombus formation. Moreover, elevated CRP produced as a result of infection (eg, by *Chlamydia*) may prime blood monocytes and elevate responses to LPS, thereby exacerbating existing inflammatory lesions and provoking thrombosis. The balance of these mediators, and their interaction with age, sex, circulating levels of CRP, estrogen replacement therapy, levels of anticoagulants,[31] and fibrinolytic factors, may contribute to the variable outcome of plaque rupture and thrombosis in coronary syndromes.

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