COMBINED EFFECT OF INFLAMMATORY C-REACTIVE PROTEIN AND HYPERGLYCEMIA ON ENDOTHELIAL CELL DYSFUNCTION

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Summary

Data from literature show that endothelial dysfunction is a key factor in atherogenesis; it is characterized by a reduction of the bioavailability of nitric oxide (NO), whereas endothelium-derived contracting factors are increased leading to an impairment of endothelium-dependent vasodilation. The main causes of NO reduced bioavailability are its interaction with reactive oxygen species leading to reactive nitrogen species (RNS) formation, and the decreased capacity of endothelial nitric oxide synthase (eNOS) to produce NO. Inflammation is another end-result of endothelial dysfunction. Our aim was to evaluate the effect of C-reactive protein (CRP), known marker of inflammation, on NO bioavailability and endothelial-derived inflammatory molecules. To this purpose, we prepared pure CRP and incubated it with human umbilical vein endothelial cells (HEC) in DMEM with 5 mM or 25 mM glucose. We determined the expression of eNOS, the levels of NO and intracellular RNS. Results showed that CRP inhibits the gene and protein expression of eNOS and NO production, determines intracellular accumulation of RNS and up regulation of MCP-1 expression. We demonstrated that the signalling pathways activated by CRP in HEC were p38MAPK and NF-kB dependent. Addition of 25 mM glucose in the culture medium aggravates this process. In conclusion, CRP combined with hyperglycemia may contribute to accelerated atherosclerosis in diabetes by inducing endothelial cell dysfunction.

Keywords: CRP, endothelial cells, eNOS, MCP-1, nitric oxide

Introduction

Inflammation has a pivotal role in atherosclerosis and a key early step is endothelial dysfunction. Recently published data demonstrate the association of C-reactive protein (CRP), the archetype marker of inflammation, with atherogenesis and endothelial cell (EC) dysfunction (Agrawal et al., 2010; Devaraj et al., 2009). The vascular endothelium is a semipermeable, selective barrier for blood molecules, which regulates vascular tone and exerts anticoagulant, antiplatelet and fibrinolytic properties. The maintenance of vascular tone is accomplished by the balance between various dilator and constrictor factors, among which nitric oxide (NO) is the major vasodilator molecule. In EC, NO is generated by NO synthase (eNOS) localized in the plasma membrane caveolae. Endothelial dysfunction is characterized by a reduction of the bioavailability of NO, whereas the endothelium-derived contracting factors are increased, leading to an impairment of endothelium-dependent vasodilatation. This phenomenon is accompanied by EC activation, characterized by the secretion of pro-inflammatory and pro-coagulant factors which favour the atherosclerotic process. Chemotaxis and entry of monocytes into the subendothelial space is promoted by monocyte chemoattractant protein-1 (MCP-1), interleukin-8 (IL-8) and fraktalkine. Besides mediating endothelium-dependent vasodilatation, NO is able to inhibit leukocytes adhesion and infiltration by inhibiting MCP-1, vascular cell adhesion molecule-1 (VCAM-1) and RANTES (Aiakawa et al., 2002; Kashiwagi et al.,...
Studies in rabbit models of hypercholesterolemia demonstrate that chronic inhibition of NO synthesis causes marked acceleration of the development of vascular dysfunction and intimal lesions. In addition, apoE KO mice, animals which develop spontaneous atherosclerosis, display attenuated NO-mediated vasodilation, have more rapid progression of atherosclerosis when subjected to either long-term eNOS antagonists or genetic eNOS deficiency (Mineo et al., 2006).

Inflammation plays a critical role in atherogenesis. CRP is a marker of inflammation and has been shown in numerous prospective studies to predict cardiovascular events (Ridker, 2007). CRP is a member of the pentraxin family of proteins. It comprises 5 noncovalently associated protomers arranged symmetrically around a central pore and has a molecular weight of 118 kDa. It is a nonglycosylated protein in humans and the gene has been mapped to chromosome 1. CRP has a half-life of about 19 hours (Chang et al., 2002). CRP is produced by the liver (hepatocytes) and in the cells of the atherosclerotic lesions, kidney, neurons, and alveolar macrophages. CRP is considered a biomarker capable to predict future risk of cardiovascular disease (CVD) in apparently healthy persons (Ridker, 2003; Anand et al., 2004). CRP is expressed in human atherosclerotic plaques and both vascular cells and monocytes/macrophages appear to represent a significant source of CRP in the inflammatory vessel wall. By activating the main cell types present in the atherosclerotic lesions, CRP generated within the coronary plaques may contribute to the development and progression of atherosclerosis. Data on vascular CRP regulation are scarce. Current evidence suggests that inflammatory and metabolic factors associated with diabetes, such as high glucose, adipokines, modified lipoproteins and free fatty acids may trigger CRP production by endothelial cells, smooth muscle cells and monocytes/macrophages (Mugabo et al., 2010). Fichtlscherer et al. showed that CRP is a statistically significant independent predictor of NO bioavailability in the systemic circulation in CVD patients (Fichtlscherer et al., 2004). Individuals with higher plasma CRP levels tend to be more exposed to myocardial infarctions (van Dijk et al., 2005). CRP levels are also increased in conditions that are associated with increased CVD risk, including obesity (McLaughlin et al., 2002), type I and type II diabetes (Devaraj et al., 2006; Mita et al., 2006), insulin resistance (McLaughlin et al., 2002), and hypertension (Niskanen et al., 2004). CRP gene and protein expression in arterial plaques are 10-fold higher compared with normal arteries content. Accumulating data suggest that CRP also promotes endothelial dysfunction (Verma et al., 2006). Reports using in vitro models suggest that CRP was detected in smooth muscle cells from atherogenic lesions in coronary arteries (Davidson and Rotondo, 2004). Also, it was shown that human aortic EC synthesize and secrete CRP (Venugopal et al., 2005). Most of the potential atherogenic mechanisms of CRP action are based on model systems using cultured cells, especially EC. CRP promotes monocyte adhesion and chemotaxis, increased uptake of oxidized low density lipoproteins (oxLDL) and stimulates the expression of matrix metalloproteinases in macrophages (Jialal et al., 2004; Chait et al., 2005). It was demonstrated that CRP is able to decrease NO bioavailability by different mechanisms including: NAD(P)H oxidase activation via p38MAPK pathway (Qamirani et al., 2005); inhibition of eNOS activity by eNOS dephosphorylation at Ser1177 (Mineo et al., 2005) or by uncoupling from the plasma membrane (Singh et al., 2007; Hein et al., 2009). Recently, the impairment of endothelial function exerted by CRP was related to its ability to alter eNOS protein–protein interaction toward a less active state of the enzyme (Valleggi et al., 2010).
Our hypothesis was that CRP and high glucose combine to aggravate endothelial dysfunction. The aim was to investigate the effect of human EC exposure to CRP and 25 mM glucose by determining eNOS expression, levels of secreted NO and intracellular RNS, expression of MCP-1. To assess the molecular mechanisms of CRP action, p38MAPK and NF-kB signalling pathways were investigated.

Material and methods

Reagents
Protease inhibitor cocktail, sodium orthovanadate, sodium fluoride, dihydorhodamine 123 (DHR) and total RNA isolation kit were from Sigma–Aldrich Co., USA. Reagents for cDNA amplification were from Promega Corporation, USA and Invitrogen Ltd., UK. Antibodies for eNOS, MCP-1, phospho-p65 (pp65) and all the secondary antibodies were from Abcam, UK. The antibodies for phospho-p38 MAP kinase (pp38) were from Cell Signaling Technology, Inc., USA, and β-actin was from Santa Cruz Biotechnology, Inc., USA. The enhanced chemiluminescence detection (ECL) kit was from Pierce, USA and the Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) from Invitrogen Ltd., UK. Commercial recombinant human CRP was from Calbiochem.

CRP purification
To remove NaN3 from the commercial preparation, CRP was dialyzed using a slide-a-lyzer cassette with a cutoff of 3000 Da (Pierce Biotechnology, Inc.) at 4°C, over night (Liu, 2005). Endotoxin, which can affect EC function, was removed from the CRP by using Detoxi-Gel Columns (Pierce Biotechnology, Inc.) according to the producer instructions.

Cell culture and experimental procedure
Human umbilical vein endothelial cells from the EA.hy926 cell line (HEC) were purchased from ATCC, USA. The cells were cultured in Dulbecco’s modified Eagle Medium (DMEM) with 5 mM glucose and 10% foetal calf serum (FCS). At confluence, the cells were starved in DMEM without phenol red and ferrous nitrate, with 0.2% FCS and 5 mM glucose, and then incubated 24 h with 20 µg/ml CRP in DMEM supplemented with either 5 or 25 mM glucose. After 24 h, the cells and the culture media were harvested and further analysed.

Nitric oxide assay
Nitric oxide (NO) production was determined by measuring the levels of nitrates in the cell culture medium using the Griess reaction, as previously described (Smarason et al., 1997).

Reactive nitrogen species measurement
The formation of nitrogen species was detected using the cell-permeant oxidant-sensitive nonfluorescent probe, DHR. Oxidation of DHR by two electrons yields the highly fluorescent product rhodamine 123. The determination of the nitrogen species followed the same protocol as that for the reactive oxygen species determination (Sima et al., 2010), except DHR used as fluorochrome. The excitation/emission wavelengths for DHR are 505/530 nm.

Quantification of eNOS and MCP-1 gene expression
Total RNA was isolated and reverse transcribed using MMLV-RT. The complementary DNA (cDNA) was amplified using specific primers for human eNOS, MCP-1 and β-actin (as the reference gene), in an Applied Biosystem Real-Time PCR StepOne Plus. The sequences of the specific primers are: for eNOS, sense 5’-GACGCTACGAGGAGTGGAAG-3’ and antisense 5’-TAGGTCTTGGGTTTGTCAAG-3’; for MCP-1, sense 5’-GAGCTACGAGGATGGAAG-3’ and antisense 5’-GCATGAAAGTCGCAAATGTGCAAG-3’ and for β-actin, sense 5’-GGGAATCGTGCCTTTC-3’ and antisense 5’-TGTGTTGCGTACAGGTTTCTTG-3’.
The amplification products were detected using SyBr green. The quantification of PCR products was performed using the “Fit Point Method” and expressed as arbitrary units. The gene expression was expressed relative to the values obtained for control cells.

**Evaluation of eNOS, pp38 MAP kinase and pp65 protein expression**

After exposure of HEC to CRP in DMEM supplemented with either 5 or 25 mM, the cells were processed as previously described (Toma et al., 2009) in the presence of protease inhibitor cocktail and phosphatase inhibitors (1 mM NaF and 2 mM Na3VO4). Specific primary antibodies for human eNOS, pp38MAPK, pp65 or β-actin were used. The relative protein expression was determined by densitometric analysis of the peaks using TotalLab 120 software (Nonlinear Dynamics Ltd., UK) and was expressed relative to protein of control cells.

**MCP-1 quantification in the culture medium**

After exposure of HEC to CRP, the culture medium was collected and secreted MCP-1 was assayed by Western Blot as previously described (Toma et al., 2009). MCP-1 protein was expressed relative to the total protein in the culture medium.

**Protein assay**

Protein concentration of each sample was measured with bicinchoninic acid (BCA) protein assay, using bovine serum albumin as standard.

**Statistical analysis**

Statistical evaluation was done by t-test with two-tailed distribution. P ≤ 0.05 was considered statistically significant. Data were expressed as means ± SD.

**Results**

**CRP inhibits the gene and protein expression of eNOS in HEC**

CRP incubated for 24 h with HEC in DMEM with 5 mM glucose induced a statistically significant decrease of eNOS gene expression relative to control cells (0.27 ± 0.11 vs. 1.11 ± 0.38, p < 0.05) (Figure 1A). The reduced gene expression of eNOS was accompanied by a decrease of eNOS protein in CRP-exposed HEC compared to control cells (0.57 ± 0.02 vs. 1.00 ± 0.09, p < 0.05) (Figure 1B). The concomitant exposure of HEC to CRP and 25 mM glucose induced an additional significant decrease of the gene (0.06 ± 0.03, p < 0.05) and protein (0.25 ± 0.03, p < 0.01) expression of eNOS (Figure 1A and B).

![Figure 1. CRP decreases the gene (A) and protein (B) expression of eNOS in HEC incubated for 24 h with normal (5 mM) or 25 mM glucose (HG) in DMEM.*p < 0.05, **p < 0.01, ***p < 0.001 vs. control cells; *p < 0.05, **p < 0.01 vs. CRP-exposed cells, n=3.](image-url)
**CRP modulates the nitric oxide production and intracellular accumulation of reactive nitrogen species in HEC**

The incubation of HEC with CRP in DMEM with 5 mM glucose for 24 h induced a significant decrease of NO levels in the culture medium compared to control cells (5.27 ± 0.27 vs. 6.86 ± 0.97 µM, p < 0.05). The concomitant incubation of the cells with CRP and increased glucose concentration in the medium (25 mM) intensified the effect of CRP alone (4.81 ± 0.24 vs. 5.27±0.27µM, p<0.05)(Figure 2A).

The intracellular reactive nitrogen species (RNS) levels were increased in HEC exposed to CRP compared to control cells (13386.27 ± 48.28 vs. 6926.84 ± 618.64 AFU/mg protein, p < 0.001). Addition of 25 mM glucose to CRP in the medium induced a supplementary increase of the level of RNS in HEC (16576.59 ± 127.04 vs. 13386.27 ± 48.28 AFU/mg protein, p < 0.001) (Figure 2B).

![Figure 2](image-url)

**Figure 2.** Nitric oxide (NO) levels in the culture medium (A) and intracellular reactive nitrogen species (B) in HEC exposed for 24 h to 20 µg/ml CRP in normal (5 mM) or 25 mM glucose (HG) in DMEM.*p < 0.05, **p < 0.01, ***p < 0.001 vs. control cells; #p < 0.05, ###p < 0.001 vs. CRP-exposed cells. AFU: arbitrary fluorescence units, n=3.

**CRP enhances MCP-1 expression in HEC**

CRP incubated with HEC in DMEM with 5 mM glucose for 24 h induced a 2-fold increase of MCP-1 mRNA levels relative to control cells (2.40 ± 0.47 vs. 1.04 ± 0.35, p < 0.05) (Figure 3A). CRP also increased MCP-1 protein secreted by HEC in the culture medium (9.19 ± 0.39 vs. 1.00 ± 0.04, p < 0.01). Increasing glucose in the medium to 25 mM in HEC incubated with CRP determined a more than 2-fold increase of MCP-1 protein secreted by HEC (24.30 ± 0.56 vs. 9.19 ± 0.39, p < 0.001) (Figure 3B).

![Figure 3](image-url)

**Figure 3.** CRP induces increased MCP-1 expression in HEC exposed for 24 h to normal (5 mM) or 25 mM glucose (HG) in DMEM.*p < 0.05, **p < 0.01, ***p < 0.001 vs. control cells; ###p < 0.01 vs. CRP-exposed cells, n=3.
**CRP stimulates NF-kB and p38MAPK**

To assess whether p38MAPK and NF-kB subunits’ phosphorylation is modulated by CRP, Western Blot analysis was performed. Compared to control cells, an increase of p65 (3-fold) and p38MAPK (2-fold) phosphorylation in HEC exposed to CRP in DMEM with 5 mM glucose for 24 h was observed (Figure 4A and B).

![Figure 4](image)

**Discussion**

Atherogenesis is a multifactorial disease aggravated by inflammation. CRP is a prototypic marker of inflammation and has been shown in numerous prospective studies to predict CVD. Small increases in CRP predict the likelihood of developing cardiovascular events both in diabetic and nondiabetic populations. Several studies have shown that high CRP levels are associated with impaired endothelial vasoreactivity and decreased eNOS activity in vivo (Teoh et al., 2008; Schwedler et al., 2007; Schwartz et al., 2007). In patients with type 2 diabetes, low grade inflammation is reflected by increased plasma levels of several biomarkers of inflammation such as CRP (King et al., 2003). In addition, in apparently healthy subjects, increased levels of CRP predict the risk of developing type 2 diabetes.

It has been shown that CRP can increase the production of superoxide in cultured human aortic endothelial cells, inducing endothelial dysfunction (Venugopal et al., 2003). The hallmark of endothelial dysfunction is impaired endothelium-dependent vasodilation, the result of an imbalance between NO synthesis and the production of ROS, such as superoxide (O$_2^-$) and the even more toxic peroxynitrites (ONOO$^-$) (Harrison, 1997). It was demonstrated that CRP inhibits endothelium-dependent NO-mediated dilation in coronary arterioles by NAD(P)H oxidase production of O$_2^-$ via p38MAPK activation (Qamirani et al., 2005).

In the present study we have shown that CRP inhibits the gene and protein expression of eNOS, reduces NO production and determines accumulation of RNS in cultured human EC. In addition, CRP up-regulates MCP-1 expression and secretion. We demonstrated that the signalling pathways activated by CRP in HEC were p38MAPK and NF-kB dependent, in good agreement with Singh et al. (Singh et al., 2008).

It is known that under hyperglycemic conditions the oxidative stress is enhanced, NF-kB and TNF-α are activated, IL-6 and adhesion molecules are increased, and NO-mediated vasodilation is impaired (Dandona et al., 2007). Our data show that combined exposure of human EC to CRP and high glucose concentration aggravates the endothelial dysfunction induced by either of them alone.
Conclusions
In conclusion, we brought evidence that CRP and high glucose act in human endothelial cells by increasing oxidative stress, decreasing nitric oxide bioavailability and inducing adhesion molecules expression. Their combined attack aggravates endothelial cell dysfunction, thus contributing to the induction of accelerated atherosclerosis in diabetes.

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