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C-reactive protein (CRP) up-regulates expression of receptor for advanced glycation end products (RAGE) and its inflammatory ligand EN-RAGE in THP-1 cells: Inhibitory effects of atorvastatin

Nitin Mahajan [a], Ajay Bahl [b], Veena Dhawan [a,*]

[a] Department of Experimental Medicine & Biotechnology, Postgraduate Institute of Medical Education & Research (PGIMER), Chandigarh, 160012, India
[b] Department of Cardiology, Postgraduate Institute of Medical Education & Research (PGIMER), Chandigarh, 160012, India

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Abstract

Background: Receptor for advanced glycation end products (RAGE) may play an important role in inflammatory processes and endothelial activation. Extracellular newly identified RAGE binding protein (EN-RAGE), natural pro-inflammatory ligand for RAGE. The role of C-reactive protein (CRP) as a mediator in inflammation and atherosclerosis is the subject of recent investigations worldwide. In the present study, we investigated the effect of CRP on RAGE and EN-RAGE gene expression in THP-1 monocytic cell line. MAP kinases (ERK, p38 and JNK) were exploited as possible signaling pathways involved in the signal transduction by CRP. Further, atorvastatin was used as a therapeutic modality for modulation of these genes in the presence of CRP.
Materials and methods: Time and dose-dependent experiments were carried out in the presence of CRP. Specific MAPK pathways inhibitors were used to elucidate the signaling pathways involved. Effect of atorvastatin was also determined in the presence of CRP on the expression of these genes.
Results: Time and dose-dependent experiments revealed that, treatment of THP-1 cells with 100 μg of CRP/ml/10^6 cells for 24 h, augmented the expression of RAGE and EN-RAGE genes by 2.5–3.5 folds and 3.5–4.5 folds respectively. CRP acted via FcγRII and utilized ERK, p38 and JNK pathways to transduce signals. Atorvastatin in a dose of 20 μM, was able to attenuate up-regulation of CRP-induced genes ($p<0.01$) and effects were both dose and time-dependent.
Conclusion: Our data strongly suggests that blockade of RAGE–EN-RAGE by statins at an early stage may prevent inflammation in atherosclerosis and counteract the harmful effects mediated by CRP.

Keywords: C-reactive protein; RAGE; EN-RAGE; MAPK; Atorvastatin

1. Introduction

C-reactive protein (CRP) is a key pro-inflammatory cytokine which serves not only as a biomarker for the risk of cardiovascular disease (CVD) but also functions as an active mediator in development of atherosclerosis by promoting arterial endothelial activation and macrophage recruitment [1]. CRP induces the production of inflammatory cytokines, matrix metalloproteinases and tissue factor expression in monocytes [2,3]. In endothelial cells, CRP increases the expression of cell adhesion molecules (CAMs), chemokines, endothelin-1, receptor for advanced glycation end products (RAGE), decreases endothelial nitric oxide synthase (eNOS) expression and activity, and augments monocyte-endothelial cell adhesion [4,5]. In vessel wall, CRP promotes the migration and proliferation of vascular smooth muscle cells (VSMCs) and increases the production of reactive oxygen species (ROS) [6].

RAGE is a member of the immunoglobulin superfamily of cell surface proteins that interacts with a wide range of

* Corresponding author. Department of Experimental Medicine & Biotechnology, Research Block 'B', Postgraduate Institute of Medical Education & Research (PGIMER), Chandigarh-160012, India. Tel.: +91 172 2747585x5235; fax: +91 172 2744401.
E-mail address: veenad2001@yahoo.com (V. Dhawan).

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ligands, including advanced glycation end products, modified low density lipoproteins, amyloid fibrils, amphoterin (HMGB1) and various S100 proteins. RAGE has been documented in promoting inflammation and endothelial activation, which in turn also accelerates coronary atherosclerotic development. RAGE activation is enhanced by accumulation of its ligands in inflammatory compartments, initiating a vicious cycle causing further up-regulation of the receptor and sustained cell activation [7,8] and molecule has also been shown to be highly expressed in human atherosclerotic lesions where it co-localizes with pro-inflammatory and pro-oxidative mediators. In addition, activation of the RAGE pathway has been shown to be important in wound healing, tumor growth and metastasis, as well as in systemic amyloidosis [9].

Among various ligands for RAGE, S100A12 [also termed as extracellular newly identified RAGE binding protein (EN-RAGE)] has been the least explored in the field of atherosclerosis. EN-RAGE has a distinct role during inflammation [10,11]. Engagement of the membrane RAGE by calcium-bound EN-RAGE activates intracellular signalling cascades including mitogen activated protein kinases (MAPK) and NFkB [12]. MAPK is activated in monocytes–macrophages in a variety of inflammatory settings [13]. Notably, ERK, p38 and JNK activation in these cells results in the expression of many of the cytokines, CAMs and inflammatory molecules observed in atherosclerotic lesions [14,15].

Statins, a class of drugs which besides being 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase inhibitors, exhibit pleiotropic properties both in *in vivo* and *in vitro* conditions [16]. Statins have been shown to decrease ROS production and VSMC proliferation, enhances eNOS levels exhibit anti-thrombotic and anti-inflammatory effects [17,18] thereby, stabilize atherosclerotic plaques [19,20].

Since, CRP is present in the atherosclerotic lesions; it may actively contribute to the progression and/or instability of the atherosclerotic plaques. Therefore, in the present study, we investigated the effects of CRP in modulation of RAGE and EN-RAGE expression in THP-1 cells. MAP kinases (ERK, p38 and JNK) were exploited as possible signaling pathways involved in the modulation of these genes by CRP. The underlying mechanisms behind this relationship have not been fully elucidated. Further, atorvastatin was used as a therapeutic approach to understand whether, it influences CRP-induced effects on RAGE and EN-RAGE genes.

2. Materials and methods

2.1. Cell culture

The human leukemic monocytic cell line (THP-1) was obtained from the National Center for Cell Sciences, Pune, India and was maintained as described previously [21].

2.2. Recombinant human CRP

The commercial recombinant *E. coli*-derived CRP preparation used was supplied in 20 mmol/L Tris, 140 mmol/L NaCl, 2 mmol/L $CaCl_2$, pH 7.5, and 0.05% (wt/vol) sodium azide (236608, Calbiochem). To remove the azide from CRP, 1 mg CRP was dialyzed twice against 500 ml of the Tris/NaCl/$CaCl_2$ buffer at 4 °C. Purity of recombinant human CRP was checked by SDS-PAGE which yielded a single intact band when 2 μg sample was loaded onto 12.5% gel. Bacterial endotoxin levels were determined using E-toxate kit (ET0100, Sigma). All reagents used in the experiments had endotoxin levels <0.125 IU/ml. E-toxa clean (E9029-500G, Sigma) along-with autoclaving (121 °C for 1 h) and dry heat (250 °C for 3 h) was used to remove endotoxin contamination from glassware and water.

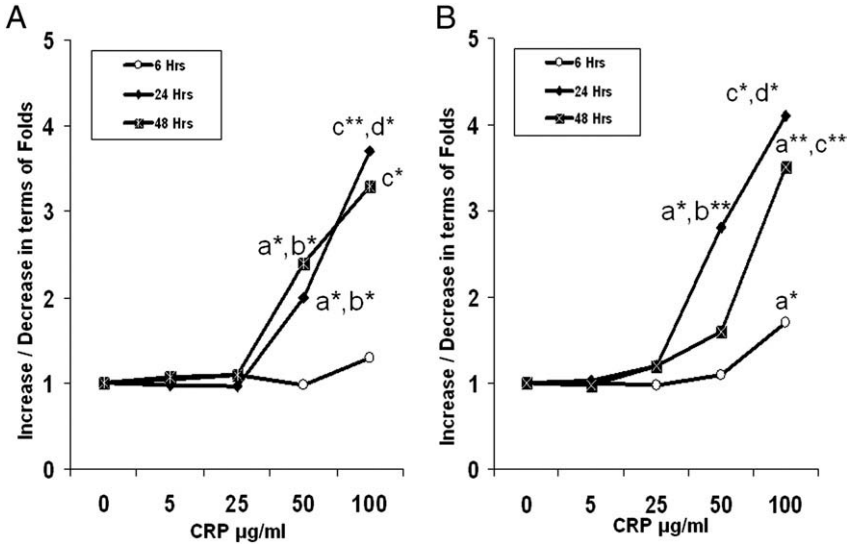

Fig. 1. Time and dose-dependent effects of C-reactive protein on expression of RAGE (A) and EN-RAGE (B) genes by THP-1 monocytic cell line by semi-quantitative RT-PCR. a=vs basal expression; *b*=25 μg vs 50 μg; *c*=50 μg vs 100 μg; *d*=24 h vs 48 h; *$p<0.05$; **$p<0.01$.

2.3. Cell viability and cell proliferation

Cell viability and cell proliferation of THP-1 cells was determined by trypan blue exclusion and cell proliferation assays (11647229001, Roche Diagnostics) respectively.

2.4. RNA extraction and RT-PCR

Semi-quantitative RT-PCR was performed to determine mRNA expression of RAGE and EN-RAGE in THP-1 cells. Total cellular RNA was isolated [21] and was further reverse transcribed to cDNA using random hexamers (K1162, Fermantas). cDNA was then amplified by PCR using human RAGE, EN-RAGE and β-actin specific primer pairs. The levels of mRNA expression of each gene were calculated as described previously [21] and expressed as percentage value or in terms of folds increase or decrease.

2.5. Effect of CRP on expression of RAGE and EN-RAGE

THP-1 cells were incubated with different doses of CRP (0–100 μg/ml) for different time intervals i.e. 0, 6, 24 and 48 h and after incubation mRNA expression of these genes were determined.

2.6. Involvement of receptors (FcγRs)

FcγRs are the major receptors for CRP in leukocytes. To determine the role of these receptors in the regulation of RAGE and EN-RAGE by CRP in THP-1 cells, blocking experiments were performed using antibodies to these receptors. For this, THP-1 cells were incubated with 50 and 100 μg/ml of FcγRI (CD64) (AF1257, R&D Systems) and FcγRII (CD32) (MAB1330, R&D Systems) monoclonal antibodies separately, 2 h prior to stimulation with the CRP and mRNA expression of RAGE and EN-RAGE genes were determined at 24 h.

2.7. Involvement of ERK, p38 and SAPK/JNK signaling pathways

For elucidating the signaling pathway involved in the expression of these genes by CRP, we used specific inhibitors for the ERK (PD98059, Sigma), p38 (SB203580, Sigma) and SAPK/JNK (SP600125, Sigma) pathway in the presence or absence of CRP.

2.8. Effect of atorvastatin on expression of RAGE and EN-RAGE

Time and dose-dependent experiments were performed with different doses of atorvastatin (0–20 μM) in the presence or absence of 100 μg/ml of CRP for different time periods i.e. 6, 24 and 48 h. Cells were harvested and mRNA expression of RAGE and EN-RAGE genes was determined.

2.9. Statistical analysis

Results are expressed as Mean±S.D of at least 3 independent experiments. A value of $p<0.01$ was considered as highly significant and all values of $p<0.05$ were considered as significant. All the analysis was performed using SPSS 14.0.

3. Results

3.1. Cells viability and cell proliferation

CRP (100 μg/ml) had no significant effect on the viability and proliferation of cells up to 48 h, whereas, it was cytotoxic at 72 h. However, CRP at a dose of 150 μg/ml showed a significant decrease in cell viability and proliferation of cells at 48 h. A minimal dose of CRP i.e. 25 μg/ml was able to adversely affect cells at 72 h both in terms of viability and morphology. No morphological abnormalities were found in cell cultures up to 48 h with 100 μg/ml CRP (data not shown). Based on these observations, further, we used CRP in a maximum dose of 100 μg/ml and the experiments were carried out up to 48 h only.

3.2. Effect of CRP on RAGE and EN-RAGE expression

CRP in a dose of 100 μg/ml was able to induce expression of both RAGE and EN-RAGE genes, which increased 2.5–3.5 folds and 3.5–4.5 folds respectively (Fig. 1). The maximum expression of both the genes was noted at 24 h. Even a minimal dose of 50 μg/ml of CRP was able to induce both genes in these cells, but the maximum effects were observed with a CRP dose of 100 μg/ml only. On the basis of these observations, we chose only 100 μg/ml dose of CRP, for further experiments. Also, no significant changes in the mRNA expression of both these genes were observed with different doses of CRP at 6 h except in case of EN-RAGE where a dose of 100 μg CRP was effective ($p<0.05$).

3.3. FcγRII is involved in CRP induced expression of RAGE and EN-RAGE

100 and 50 μg/ml of anti-CD32 antibody was able to block CRP-stimulated RAGE expression by 50% and 35% respectively and inhibit EN-RAGE expression by 41% and 20% respectively. Anti-CD64 antibody was less effective in blocking CRP-stimulated RAGE expression in these doses i.e. 19% and 18%, respectively, whereas, EN-RAGE expression was blocked up to 8% and 6% only. To further confirm whether, anti-CD32 antibody blocks CRP-induced RAGE and EN-RAGE expression by specific binding to FcγRII, an isotype control antibody (clone 27 to 35) for the anti-CD32 was also used. We observed that it did not block the observed stimulation of these genes. Our data demonstrated that, it is the FcγRII (CD32) receptor which is mainly involved in the CRP induced expression of RAGE and EN-RAGE.

3.4. ERK, p38 and JNK signaling pathways are involved in transduction of CRP-induced expression of RAGE and EN-RAGE

All the inhibitors reduced CRP-induced mRNA expression of RAGE as well as EN-RAGE genes which suggested that the expression of both the genes induced by CRP is dependent on three different pathways (ERK, p38 and JNK) (Fig. 2). Since, MAPK inhibitors were dissolved in dimethyl sulphoxide (DMSO), an equivalent amount of vehicle (0.05% DMSO) was also added to the control cells. No significant differences in the expression of these genes were observed either in the absence or presence of the vehicle (data not shown).

3.5. Inhibition of CRP induced expression of RAGE and EN-RAGE by atorvastatin

Incubation of THP-1 cells with atorvastatin attenuated the expression of both these genes in a time and dose-dependent manner (Fig. 3). Maximum decrease in the expression of these genes was observed with 20 μM dose of atorvastatin at 48 h and a minimal dose of 5 μM of atorvastatin for 48 h was able to attenuate expression of both the genes. No significant alteration in the expression of these genes was observed with atorvastatin till 6 h. As atorvastatin is a lipophilic drug, it was dissolved in DMSO as a vehicle and, therefore, an equivalent amount of vehicle was also added to the control samples.

4. Discussion

Atherosclerosis has an inflammatory etiology and highly elevated CRP in patients with atherosclerosis not only serves as

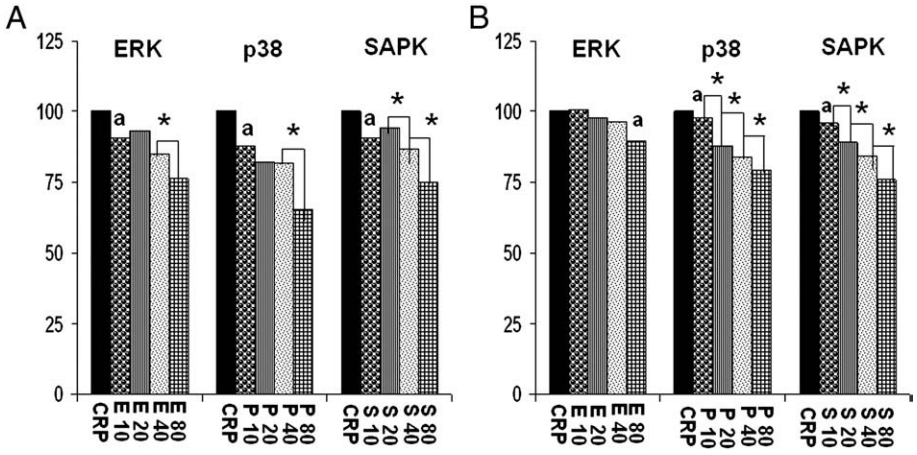

Fig. 2. Effect of different doses (0–80 μM) of inhibitors of MAPK pathways i.e. PD 98059 [ERK (E)], SB 203580 [p38 (P)], SP 600125 [SAPK (S)] on CRP up-regulated gene expression of RAGE (A) and EN-RAGE (B) by THP-1 monocytic cell line as determined by semi-quantitative RT-PCR. *a* = vs basal; **p*<0.05.

a biomarker for CVD risk but it also functions as an active mediator of atherosclerosis by its direct proatherogenic effects on the vasculature [22,23]. Among others, RAGE and EN-RAGE molecules are expressed by lymphocytes and monocyte–macrophage lineage cells in response to the inflammatory cytokines produced during the acute-phases of inflammation.

We observed that CRP up-regulated the expression of RAGE in a dose and time-dependent manner in THP-1 monocytic cell line. The augmented expression of RAGE in the presence of CRP as observed in this study, further corroborates the concept that RAGE participates as one of the mechanism that may assist in explaining its role in various diseases characterized by underlying inflammation. In an isolated report by Zhong et al. [5], expression of RAGE in human endothelial cells was found to be increased in the presence of CRP and suggested that CRP enhances the binding ability of RAGE to its ligands.

Our results demonstrated that CRP was able to augment mRNA expression of both RAGE and EN-RAGE genes. Hofmann et al. [10] reported the induction of EN-RAGE expression by LPS and observed a bell shaped curve for the expression of EN-RAGE. The expression increased initially, reached a plateau and subsequently decreased with increment in the doses of LPS. However, we could not determine the expression of these genes beyond 100 μg/ml of CRP concentration because of the adverse effects of CRP on viability and proliferation of THP-1 cells. Also, we observed maximum expression of both these genes at 24 h. Augmented expression of EN-RAGE in the presence of CRP points towards a close correlation between EN-RAGE and activation of granulocytes and monocytes under inflammatory conditions. The

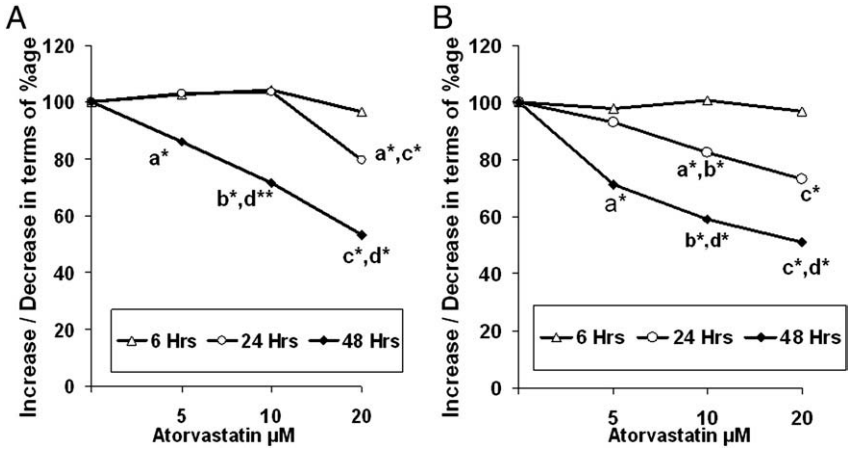

Fig. 3. Time and dose-dependent effect of atorvastatin (0–20 μM) on CRP up-regulated gene expression of RAGE (A) and EN-RAGE (B) by THP-1 monocytic cell line as determined by semi-quantitative RT-PCR. *a* = vs CRP up-regulated expression; *b* = 5 μM vs 10 μM; *c* = 10 μM vs 20 μM; *d* = 24 h vs 48 h; **p*<0.05; ***p*<0.01.

inflammatory role of RAGE and EN-RAGE interactions is well established in various chronic inflammatory disorders [10,11].

Moreover, we observed a parallel trend of increased RAGE and EN-RAGE expression in response to CRP treatment. Our data further supports the previous observations that, expression of RAGE is up-regulated in the presence of its own ligands by a positive feedback loop mechanism [9]. Basta et al. [24] have reported a positive correlation of hsCRP levels with plasma EN-RAGE in diabetic subjects. Though, ours is an *in vitro* study, we also observed a positive association between CRP treatment and increased EN-RAGE expression which was dose-dependent. Human recombinant S100A12 has been reported to be chemotactic for neutrophils and monocytes both *in vitro* and *in vivo* [25,26]. The parallel trend observed for the increased expression of this receptor and its ligand represents an attractive model to explain how RAGE and its pro-inflammatory ligand contribute to the pathophysiology of various inflammatory diseases including atherosclerosis [27]. On the contrary, Kim et al. [28] did not observe any correlation between these two genes in mice infected with *M. leprae*. Kawahara et al. [29] recently demonstrated it was shown that CRP up-regulates the expression of HMGB1 via p38 MAPK pathway and further suggested its role in inflammation. The excessive expression of these genes at sites of inflammation may feasibly form a positive feedback loop which may further lead to recruitment of more inflammatory cells and thus aggravate the underlying inflammation. Hasegawa et al. [30] have demonstrated that levels of mRNA/protein of S100A12 were enhanced by another pro-inflammatory molecule i.e. IL-6 in cultured THP-1 cells.

In patients with chronic CAD, plasma CRP concentrations are increased only mildly, the levels being < 10 μg/ml and mild inflammation and viral infections are shown to cause elevation in CRP levels to 40 μg/ml [31]. According to our *in vitro* data, these elevations may not be enough as to evoke a heightened expression of RAGE and EN-RAGE gene by THP-1 cells. Considering that CRP is accumulated in atherosclerotic lesions and may be expressed by cells in the lesions, it is possible that CRP concentration in atherosclerotic plaques could be much higher than that in the plasma [32]. Therefore, it is likely that local CRP levels in atherosclerotic lesions are sufficient to stimulate RAGE and EN-RAGE expression by macrophages and thus supports our findings.

FcγRII and RI are the high and low affinity receptors for CRP in THP-1 cells [3] and our data clearly demonstrates that FcγRII actively participates in up-regulation of RAGE and EN-RAGE by CRP in THP-1 cells. Our study is in accordance with earlier observations, where authors reported that CRP engages Fc gamma II receptor for up-regulation of different genes [3,4,29].

MAPKs are likely to play crucial roles in the genetic response of many components of the cardiovascular system to the disease process [15]. CRP significantly up-regulates expression of RAGE and EN-RAGE by macrophages, thus highlights an important pathophysiological role of CRP in inflammatory conditions. We demonstrated activation of all the three signalling pathways (ERK, p38, JNK) in modulation of RAGE and EN-RAGE by CRP in THP-1 cells. HMGB, another ligand of RAGE has also been shown to be up-regulated by CRP via p38 MAPK pathway [29]. Also, previous studies have shown that CRP by activating either of ERK, p38 or JNK, modulates the genes involved in inflammation and vascular remodelling [3,4]. Therefore, all these findings cumulatively suggest MAPK as an obvious therapeutic target for the diseases involving RAGE and EN-RAGE.

Observations obtained so far, pinpoint that RAGE and EN-RAGE axis co-operates with pro-inflammatory cytokines in an autocrine, paracrine and endocrine manner at the atherosclerotic lesion and contributes in the disease progression in these patients. Next we explored the effect of atorvastatin, on the CRP-induced genes in these cells.

Atorvastatin treatment significantly attenuated CRP-induced RAGE and EN-RAGE expression in THP-1 cells. As far as the literature is concerned, evidence related to the effect of atorvastatin on modulation of these genes is lacking. Also, in a similar dose as used in the present study, atorvastatin has been shown to inhibit the expression of resistin [33], lipoprotein and endothelial lipase activity [34]. Though other statins are shown to attenuate CRP-induced expression of certain candidate genes involved in inflammation and atherosclerosis [4,35,36], data with atorvastatin and on RAGE and EN-RAGE is still lacking. Our data highlights important anti-inflammatory properties of atorvastatin which may be mediated by non-steroidal products of the mevalonate pathway. Statins have been shown to inhibit the MAPK pathways in aortic endothelial cells (AoEC) [37], aortic SMC [38] and cardiac myocytes [39].

Findings of the present study provide sufficient evidence for potential links among CRP and RAGE–EN-RAGE axis in atherosclerotic disease. We used a monocytic cell line, the cells of which are often used as fair substitutes for simulating human monocytes–macrophages in analyzing the production of inflammatory mediators/cytokines, including MMPs, in response to various inflammatory stimuli [40].

To the best of our knowledge, this is the first report to demonstrate that CRP up-regulates the expression of RAGE and its ligand EN-RAGE in THP-1 cells. It acts via FcγRII and utilizes MAPK i.e. ERK, p38 and JNK pathways to transduce signalling. Our findings also, suggest that CRP plays a potential role in the induction, amplification, and prolongation of inflammatory response in atherosclerosis. RAGE and EN-RAGE molecules can be viewed as potential drug targets for statins and statin like drugs in the prevention of CAD.

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The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [41].

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