Low-level C-reactive protein levels exert cytoprotective actions on human podocytes

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Abstract

Background. Albuminuria and elevated C-reactive protein (CRP) levels are common manifestations of many inflammatory diseases. Cardiovascular-based drugs, with secondary anti-inflammatory actions, such as angiotensin-converting enzyme-inhibitors are able to reduce both proteinuria and CRP levels, raising the question of whether CRP directly influences the processes that result in proteinuria. As proteinuria is thought to be induced as a result of podocyte dysfunction, we investigated whether there is a pathomechanistic link with CRP.

Methods. Podocytes were analysed for evidence of endogenous CRP production in response to inflammatory agents. In addition, they were incubated in the presence of various concentrations of exogenous CRP and analysed for evidence of a response to treatment.

Results. Our results demonstrated that inflammatory agents such as macrophage-conditioned medium and interleukin-1β induced the expression of CRP messenger RNA in podocytes. However, they were unable to induce CRP protein. Stimulation of podocytes with exogenous CRP demonstrated that 10 μg/mL CRP induced a low but significant level of interleukin-6 secretion. Tumour necrosis factor α, however, was not detected. CRP did up-regulate the expression of the slit diaphragm proteins nephrin and CD2AP, as well as the structural proteins ezrin and podocalyxin-like protein-1, proteins known to be involved in signalling via the phosphotidylinositol-3 (PI-3) kinase pathway. CRP exposure reduced caspase-3 enzyme activity and up-regulated the expression of the anti-apoptotic protein Bcl-2. In the presence of the PI-3 kinase inhibitor LY294002, the ability of CRP to suppress caspase-3 activity was significantly reduced.

Conclusions. Taken together, these data suggest that rather than inducing podocyte damage, CRP may be a survival factor for podocytes by maintaining their structural integrity and initiating a survival cascade, which may facilitate podocyte recovery from injury.

Keywords: apoptosis; C-reactive protein; PI-3 kinase pathway; podocytes; slit diaphragm proteins

Introduction

C-reactive protein (CRP) is a highly conserved prototypical acute phase reactant, which functions to facilitate the innate immune system and limit tissue damage in autoimmune reactions [1, 2]. Consistent with this role, plasma concentrations of CRP can increase from basal levels of 1 μg/mL (median concentration 0.8 μg/mL) to >1000 μg/mL within 24–48 h in response to acute inflammation or injury and decrease rapidly to baseline levels following resolution of disease. However, it is the chronically raised levels of CRP (>10 μg/mL) that are indicative of an underlying pathology. It is commonly accepted that raised CRP levels are characteristic of atherosclerotic cardiovascular disease, an inflammatory disease of vessel walls. Evidence suggests that such cardiovascular disease occurs, at least in part, as a result of CRP-induced endothelial cell dysfunction. These effects include increased expression of adhesion molecules [3], decay-accelerating factor [4] and secretion of chemokines [5], reduced expression of eNOS [6], reduced secretion of von Willebrand factor [7], inhibition of cell proliferation, reduced cell viability [8] and increased apoptosis [9]. However, some of these effects have been ascribed to lipopolysaccharide (LPS) and/or azide, agents known to be present in commercial CRP preparations [10, 11].

Albuminuria, like raised CRP levels, is often found in association with inflammatory disease and is also thought to be a consequence of the generalized endothelial dysfunction that occurs throughout the vasculature. Agents such as renin angiotensin system blockers [12], HMG-CoA reductase inhibitors [13, 14] and thiazolidinediones [15, 16] have been
shown, in addition to their specific pharmacological actions, to reduce CRP levels, proteinuria and cardiovascular risk. Although the mechanism of these effects has not been fully delineated these agents are known to have well-described anti-inflammatory properties.

Since proteinuria results from podocyte dysfunction, this begs the question whether there is a direct pathogenic link between podocytes, CRP and albuminuria. The aim of the study therefore was to examine whether podocytes produce and/or respond to CRP and whether the nature of the response could contribute to the induction of proteinuria.

Materials and methods

Unless otherwise stated all chemicals and reagents were obtained from Sigma (Poole, Dorset, UK).

Cell culture

The human podocyte line used in these experiments expresses a temperature-sensitive SV40 promoter that allows proliferative undifferentiated growth at 33°C and differentiated growth at 37°C. The cells were grown to confluence in RPMI 1640 containing 10% fetal calf serum, 100 μg/mL penicillin (Invitrogen, Paisley, Scotland, UK) and 100 μg/mL streptomycin (Invitrogen). 2 mM glutamine (Invitrogen). ITS supplement (insulin/selenium/transferin) (Invitrogen). The cells were then transferred to 37°C and allowed to differentiate for at least 10 days. The cell lines have previously been extensively characterized and have been shown to express a number of podocyte-specific differentiation markers [17].

C-reactive protein

Native CRP was derived from ascite fluid (Biodesign, Oxford, UK). Monomeric CRP (mCRP) was made by heating the native isoform for 70°C for 1 h [18].

In vitro experimental conditions

For most of the experiments, differentiated podocytes were exposed to native CRP at concentrations of 1 and 10 μg/mL, very approximately corresponding to moderate and elevated risk of developing heart disease. However, for some experiments, cells were exposed to additional concentrations of CRP, i.e. 0.1, 0.5, 1.0, 10 and 50 μg/mL. Podocytes were also exposed to mCRP as it has previously been reported that the monomeric form may be the biologically active form [19]. In addition, some cells were also concomitantly exposed to macrophage-conditioned medium (MCM), IL-1β (10 ng/mL) (R&D Systems, Oxford, UK), LPS (1 μg/mL) or sodium azide (15 mM). Cells were stimulated in serum-free conditions for 24 h to avoid the confounding effects of serum-borne CRP.

Preparation of MCM

MCM was prepared from U937 cells as previously described [20].

Reverse transcription-polymerase chain reaction

RNA was extracted from cells using TRizol (Invitrogen) according to the manufacturer’s instructions. Aliquots of 0.5 μg total RNA were reverse transcribed using an AMV Reverse Transcription System according to manufacturer’s instructions (Promega, Southampton, UK). The resulting complementary DNA was amplified using Platinum®/Taq DNA Polymerase High fidelity (Invitrogen) and 50 pmol of specific sense and antisense primers. Thermostrapping conditions were optimized for each primer set.

CRP was then amplified using specific primers: sense 5'-TCCTAGTGC-CAACAAAGACAAAGCA-3' and antisense 5'-AACACTTTCCGCTTG-CACTTCATA-3' [21] and glyceraldehyde 3-phosphate dehydrogenase sense 5'-ACCGATTGTGCTGATTGGG-3' antisense 5'-TGAT-TTTGAGGATCTCG-3' [22].

Protein preparation

Proteins were prepared either by TRizol extraction as directed by the manufacturer or by scraping the cells into 750 μl reducing sample buffer. Protein lysates in sample buffer were not usually assayed for protein but membranes were reprobed for β-actin to normalize for loading. However, when it was necessary to measure the protein levels of cell lysates, the cells were taken up in reducing sample buffer (minus bromophenol blue) and the protein concentrations determined by Bio-Rad DC-DC assay kit according to the manufacturer’s instructions.

Protein assay

Protein concentrations of samples prepared by the TRizol method were determined by Bio-Rad DC protein assay (Bio-Rad, Hertfordshire, UK) using bovine serum albumin (BSA) standards according to the manufacturer’s instructions for equal loading onto gels. Typically, 20-μg protein were added per well for sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis.

Protein concentrations of samples prepared in reducing sample buffer (minus bromophenol blue) were determined by the Bio-Rad DC-DC protein assay using BSA standards according to the manufacturer’s instructions.

Western blotting

Equal volumes of podocyte proteins were mixed with non-reducing sample buffer for CRP protein determination or reducing buffer for other proteins, boiled for 5 min then resolved on SDS-polyacrylamide gels of appropriate pore size. The gels were then blotted onto nitrocellulose membranes for 1 h at 100 V. The membranes were blocked in 5% milk protein solution in Tris-buffered saline containing 0.05% Tween (TTBS) saline after which they were washed and incubated with appropriate primary antibody overnight at 4°C. After washing, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. Protein bands on the membrane were detected using the SuperSignal West Pico ECL reagent (Pierce, Rockford, IL). Bands were quantified on a Bio-Rad GS 700 Imaging Densitometer.

Primary antibodies used in western blotting were as follows: rabbit anti-CRP (Calbiochem, Nottingham, UK), rabbit anti-nephrin, mouse anti-Fcγ-R1 (CD64) and RII (CD32), mouse anti-human podocalyxin-like 1, mouse anti-Bcl-2, mouse anti-caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-β-actin, rabbit anti-podocin, rabbit anti-CQ2AP (Abcam, Cambridge, UK), rabbit anti-phospho AKT, rabbit anti-total AKT (Cell Signalling Technology, Beverly, MA).

The following secondary antibodies were used in western blotting: horseradish peroxidase-conjugated goat anti-rabbit IgG (DakoCytomation, Glostrup, Denmark), horseradish peroxidase-conjugated rabbit anti-mouse IgG.

CRP enzyme-linked immunosorbent assay

The 96-well microtitre plates (Nunc, Thermo Fisher Scientific, Loughborough, UK) were coated with 70 μL rabbit anti-CRP antibody (5 μg/mL) (Calbiochem) in 50 mM carbonate buffer pH 9.6 overnight at 4°C. The plates were washed four times with wash buffer [phosphate-buffered saline (PBS) + 0.1% Tween 20]. Non-specific binding sites were blocked with 100 μL well solution of 2% BSA in wash buffer for 1 h at room temperature. Fifty microlitres CRP standards from 300 to 4.7 ng/mL [serial dilutions of CRP derived from human ascite fluid (Biodesign)] or samples (culture supernatants) were incubated for 2 h at room temperature. The plates were then washed and incubated with a monoclonal anti-CRP antibody (AbCam) at a dilution of 1/750 for 2 h at room temperature. After a further four washes, the plates were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG for 1.5 h at room temperature. Colour development was carried out using OPD (Invitrogen) as chromogen with H2O2 as the substrate. Following colour development, the reaction was stopped using 1 M H2SO4 and optical densities were measured at 492 nm on a Titertek Multiscan Plus (Titertek Instruments, Huntsville, AL).

IL-6 and tumor necrosis factor α enzyme-linked immunosorbent assay

IL-6 and tumor necrosis factor (TNFα) enzyme-linked immunosorbent assays (ELISAs) were carried out according to manufacturer’s recommendations (Duoset R&D systems).

Measurement of caspase-3 activity

Following treatment of cells with CRP in a 25-cm² flask, the cells were scraped into their culture medium (5 mL) on ice. The medium was then centrifuged at 3000 x g for 5 min. The supernatants were discarded and the pellets resuspended in 100 μL lysis buffer (Tris-acetate buffer pH 7.4) and homogenized. The lysates were then centrifuged at 11, 600 x g for 10 min at 4°C. The supernatants were carefully removed and analysed for protein concentration. Caspase-3 activity was detected by a modified fluorometric CaspASE™ Assay System (Promega). This assay is based on the ability of caspase-3 to cleave the fluorogenic substrate Ac-DEVD-7-amino-4-methyl
coumarin (AMC). The specificity of the assay was determined using the caspase-3 inhibitor Ac-DEVD-CHO. The fluorescence of this reaction was monitored at 360 nm excitation and 460 nm emission using the Mx 4000™ Multiplex qPCR System (Stratagene). Fluorescence intensity was calibrated against standard concentrations of AMC. Caspase-3 activity was expressed as picomoles AMC per minute per microgram protein at 30°C.

Immunohistochemistry
Four hundred microlitres of a cell suspension (10⁴ per well) were plated into each chamber of an eight-well Permanox slide (Lab Tek®). The cells were incubated at 33°C for 2 days after which they were transferred to 37°C to allow them to differentiate. The podocytes were stimulated with 0, 1, 10 or 50 μg/mL CRP in serum-free medium for 24 h after which the medium was aspirated and the cells washed three times with PBS. The cells in each well were then fixed with 400 μL of 4% sucrose and 2% paraformaldehyde in PBS for 10 min. After three washes with PBS, the cells were permeabilized with 0.3% Triton X-100 in PBS for 10 min. After a further three washes, the cells were incubated for 30 min with a blocking solution (4% fetal calf serum, 0.1% Tween 20 in PBS (200 μl/well)). The podocytes were then stained with FITC-conjugated phalloidin (Sigma) (5 μg/mL in 0.1% Tween 20 in PBS (30 μl/well)) for 50 min. Finally, the slides were washed in deionized water before covering the stained cells with a coverslip with a few drops of fluorescent mounting medium (DakoNyctomation). The slide was allowed to dry for 30 min in the dark before being examined using confocal microscopy (Olympus FV 1000 Confocal laser scanning microscope).

Depletion of CRP
Protein-A-insoluble cell suspension (100 μl) was washed twice in PBS. Forty microlitres of a 1:4 dilution of polyclonal anti-CRP antibody was added to the protein A and incubated at room temperature for 2 h. The protein A was then washed three times with PBS. Twenty microlitres of a 1:4 dilution of polyclonal anti-CRP antibody was added to the protein A and incubated overnight at 4°C. The Protein A suspension was spun down and the ‘CRP depleted’ supernatant was retained.

In addition, 1 μg/mL CRP was added to podocytes in the presence of 10 μL polyclonal anti-CRP antibody (Calbiochem).

Statistics
Results are shown as means ± SEMs. A t-test was carried out to determine statistical significance between means. Where multiple comparisons between means were determined, an analysis of variance with Bonferroni correction was applied. Statistical significance was defined as P < 0.05. Representative western blots are presented. However, where appropriate, the histograms represent the densitometry and statistics carried out on all blots.

Results
Growth of podocytes in serum-free medium
In order to assess the viability of podocytes cultured in serum-free medium over time, we performed a time course from 6 to 96 h. A protein assay was carried out on the cell monolayers to assess their protein content. The data demonstrated that the protein content of the cell monolayer decreased with time (Figure 1). We therefore carried out all assays at the 24 h time point.

Effect of cytokines on the production of CRP by podocytes
Podocytes were treated with the pro-inflammatory cytokine IL-1β (10 ng/mL) or with MCM both of which are known to induce the production of CRP in human aortic endothelial cells [23]. Both IL-1β and MCM induced the expression of CRP messenger RNA (mRNA) in podocytes (Figure 2) but we were unable to detect the expression of CRP protein in culture supernatants or cell protein extracts by western blotting or ELISA at Day 1 or Day 3 (data not shown). To examine whether the observed effects were due to contamination with LPS or azide, podocytes were also stimulated with 1 μg/mL LPS and 15 mM sodium azide [10, 11]. These agents were unable to induce either CRP mRNA or protein in podocytes (data not shown).

Effect of CRP on podocyte cytokine production
In order to examine the effect of exogenous CRP on podocytes, CRP was added to podocyte cultures for 24 h. Podocytes were also stimulated with MCM or IL-1β (as positive controls). After 24 h, the podocyte culture supernatants were analysed for IL-6 and TNFα.

MCM and IL-1β induced podocyte IL6 secretion into culture supernatants. However, although CRP at 1 μg/mL (native and monomeric) had no effect, CRP at 10 μg/mL induced a small but significant rise in IL6 levels (P < 0.03 versus CRP1 and CRPm) (Figure 3). Only MCM, but not CRP or IL-1β, induced TNFα secretion by podocytes (Figure 3).

Podocytes and FcγR receptor expression
For CRP to exert an effect on podocytes, it would have to bind to a receptor. CRP is known to engage FcγR receptors on immune cells and injured endothelial cells [24–27]. We therefore carried out western blotting to determine whether podocytes expressed these receptors. Our data showed that human podocytes constitutively expressed FcγRI (Figure 4). In contrast, FcγRII receptor was barely expressed in control
cells but was up-regulated following treatment with CRP (Figure 4) thus making both FcγRII and II possible candidate receptors for active interaction with CRP on podocytes. FcγRIII receptors have also been shown to bind mCRP in human neutrophils [28]. However, we were unable to detect either message or protein for these receptors on cultured human podocytes under any conditions (data not shown).

CRP and slit diaphragm protein expression

As inflammatory conditions are associated with proteinuria and since the podocyte slit diaphragm complex is thought to play a crucial role in the development of proteinuria, both structurally and via signalling pathways [29], we examined the effects of CRP on the expression of slit diaphragm proteins nephrin, podocin and CD2AP.

Western blotting demonstrated that expression of nephrin protein was significantly up-regulated by 10 μg/mL CRP and mCRP (P < 0.05 and P < 0.009, respectively) (Figure 5). CD2AP was up-regulated only by treatment with 1 μg/mL CRP (P = 0.008), while podocin expression remained unaffected by CRP (Figure 5).

CRP and structural protein ezrin and podocalyxin-like protein expression

As well as being a molecule pivotal in maintaining the podocyte cytoskeletal structure, ezrin has also been reported to signal cell survival [30]. Western blotting demonstrated that ezrin protein was up-regulated following treatment with 1 μg/mL CRP (P = 0.001) (Figure 6).

Linked to the actin cytoskeleton via ezrin is podocalyxin-like protein, a major sialoglycoprotein of the polyanionic coat required for the maintenance of podocyte structure and function. Our results showed that exposure of podocytes to 1 μg/mL CRP resulted in an up-regulation of podocalyxin-like protein over control levels (P < 0.001) (Figure 6).

CRP and apoptosis

Accumulating data suggests that an inability to replace podocytes lost by apoptosis is an early step in the pathological progression to proteinuria and glomerulosclerosis. Given that podocytes are terminally differentiated cells with limited regenerative capacity, prevention of podocyte apoptosis is critical for maintenance of podocyte number and glomerular function. For this reason, we examined whether CRP played a role in podocyte apoptosis.

Western blotting demonstrated that CRP reduced the intensity of the 24- and 17/19-kD active caspase-3 subunits expressed in control cells (Figure 7).

Bcl-2 is an anti-apoptotic protein known to inhibit the release of cytochrome c from mitochondria [31]. Bcl-2 is released from the pro-apoptotic protein Bad once the latter has been inactivated by phosphorylation with pAkt (a downstream target of PI-3 kinase) [32]. It is also transcriptionally regulated in its own right [33]. Western blotting demonstrated that Bcl-2 was up-regulated over control levels in podocytes following treatment with CRP (P < 0.05 versus medium) (Figure 8).

CRP and PI3 kinase

As some of the slit diaphragm proteins are known to signal via the PI-3 kinase/Akt signalling pathway and since their expression had been up-regulated by CRP, we wished to determine whether the PI-3 kinase survival pathway could be involved in the anti-apoptotic actions of CRP. To this end, podocytes were incubated with CRP in the presence or absence of the PI-3 kinase inhibitor LY294002 (LY) for 24 h.

Akt is a downstream signalling molecule of the PI-3 kinase pathway. Treatment of podocytes with CRP in the presence of LY (13 μM) resulted in a complete inhibition of Akt activation (phosphorylation) as observed by western blotting (Figure 9a). In over 20 assays, Akt activation (phosphorylation) in the presence of CRP was generally increased.
These data, however, did not reach statistical significance, as on some occasions we observed that Akt phosphorylation was decreased in the presence of CRP. A fluorometric CaspACE/C228 assay not only confirmed that CRP reduced caspase-3 activity but further demonstrated that PI-3 kinase inhibition reduced the suppressive effect of CRP on caspase-3 activity (*P < 0.03 CRP1 versus CRP1 LY and medium) (Figure 9b) implicating a role for CRP in activating the PI-3 kinase pathway. However, surprisingly, addition of LY294002 appeared to reduce the caspase-3 activity in control (non-CRP treated) cells (†P < 0.05 versus med).

**Fig. 5.** Effect of CRP on slit diaphragm protein expression. Western blots showing expression of nephrin, podocin and CD2AP in podocyte cell lysates in response to 1 or 10 μg/mL CRP and 1 μg/mL mCRP or medium alone (nephrin: *P = 0.005, **P < 0.001 versus med, n = 3–9; CD2AP: *P = 0.008, n = 3). β-Actin was used as a loading control. Representative blots from three to five experiments are shown—histograms show the densitometric data from all blots expressed as ratios over β-actin.

**Fig. 6.** Effect of CRP on ezrin and podocalyxin-like protein-1 expression. Western blots showing expression of structural proteins ezrin and podocalyxin-like protein-1 in podocyte cell lysates in response to 1 or 10 μg/mL CRP and 1 μg/mL mCRP or medium alone (ezrin: *P < 0.01 versus med, n = 5; podocalyxin: *P < 0.001, n = 3). β-Actin was used as a loading control. Representative blots are shown—histograms show the densitometric data from all blots expressed as ratios over β-actin.
When the effects of CRP in the presence of LY on caspase-3 protein expression were examined by western blotting, we unexpectedly found that the intensities of the 24- and 19/17-kD active subunit bands were markedly reduced. The control cells (no CRP) appeared to exhibit a greater down-regulation of these caspase-3 activated subunits than cells treated with CRP (Figure 10). However, although not always detected by western blotting, the expression of the 12-kD active subunit of caspase-3 was increased in the presence of the PI-3 kinase inhibitor (Figure 10). This may account for the overall increase in caspase-3 activity measured in the cell lysates from cells treated with CRP and LY (Figure 9b).

Specificity of CRP

In order to assess whether the observed effects of CRP were specifically due to the acute phase protein itself and not a contamination product of the ascites fluid preparation (other than azide or LPS), the ascites fluid was depleted of CRP using anti-CRP and protein A. In addition, anti-CRP antibody was added to the podocyte cultures in combination with CRP (1 μg/mL). The effects of CRP depletion and anti-CRP antibody on the expression of caspase-3 were examined by western blotting. The results demonstrated no difference in
Podocyte caspase-3 expression in response to depleted CRP or CRP in the presence of anti-CRP polyclonal antibody compared to control cells in serum-free medium, strongly suggesting that the observed effects were specific to the CRP protein itself (Figure 11).

Dose response of CRP

In the current study, the doses of CRP chosen were designed to approximately reflect normal (1 μg/mL) and chronically raised levels (10 μg/mL). In order to ascertain the extent of the dose effect, we exposed podocytes to a range of doses of CRP, i.e. 0.1, 0.5, 1.0, 10 and 50 μg/mL CRP. Western blotting demonstrated that the effects of CRP on caspase-3 protein expression appeared to follow a J shape curve with the 1 μg/mL dose exerting maximum suppression of serum-free medium-induced caspase-3 expression and 50 μg/mL CRP inducing greater activation than medium alone (Figure 12a).

Nephrin expression showed a similar pattern but with nephrin expression peaking over the 0.5–10 μg/mL dose range (Figure 12b).

Effects of CRP on podocyte morphology

In order to determine what effect CRP treatment had on podocyte cell morphology, podocytes were incubated with increasing concentrations of CRP for 24 h in serum-free medium. The cells were then stained with FITC-conjugated phalloidin. Examination of the slides by confocal microscopy demonstrated that there was no significant effect of any dose of CRP used (0, 1, 10 and 50 μg/mL) on the podocyte actin cytoskeleton in the 24-h time frame studied (Figure 13).

Discussion

Both proteinuria and inflammation are conditions associated with elevated circulating CRP levels. However, whether chronically raised CRP levels are directly involved in the pathophysiology of proteinuria and renal disease or whether they are a marker of a continuing disease process is still a topic of controversy. The current study aimed to ascertain whether there was a direct causal link between raised CRP and podocyte injury, which could lead to the initiation of proteinuria.

Although the liver is known to be the major site CRP protein secretion, the kidney has also been identified as an extra-hepatic site of CRP production with renal tubular epithelial secreting this acute phase protein [34]. In the current study, we found that cultured podocytes expressed CRP mRNA although they did not appear to synthesize CRP protein under conditions, which are known to stimulate its production in endothelial cells. This was not due to a failure in the detection method as in preliminary experiments we were able to detect CRP in hepatocyte culture supernatants as well as in CRP-spiked culture medium. It is also unlikely that this was due to insufficient time for protein translation as after 3 days one would expect to see a response to treatment given the rapid increase in CRP levels in vivo following acute exposure to infection. It may be due to the absence of a secondary signal required for initiation of protein translation.

The cytokine response of podocytes to CRP was somewhat underwhelming as we were only able to detect a small increase in IL6 levels following exposure to 10 μg/mL CRP, while TNFα was not detected at all. CRP is able to engage a number of ligands depending on the context of its action. These include phosphocholine on pathogenic bacteria [35], nuclear antigens [36], C1q to activate the classical complement pathway [37] and the Fcγ receptors [38]. Studies with CRP have shown interaction with both FcγRI and RII receptors in man and mouse. The ability to engage both activating and regulatory receptors may explain why CRP can have both pro- and anti-inflammatory actions. We were able to detect both FcγRI and FcγRII receptors in podocytes, while FcγRIII receptors were not detectable at either message or protein level. However, we cannot say at this stage whether these are the receptors responsible for the observed effects of CRP in podocytes. Nephrin is a podocyte-associated protein which plays a key role in maintaining the interpodocyte slit diaphragm structure and thereby the permselective properties of the glomerular filtration barrier. Although nephrin protein expression is known to be down-regulated in patients suffering from albuminuria [39], we found that in podocytes, nephrin expression was up-regulated by the higher concentration of CRP. The nephrin/podocin/CD2AP complex not only controls the structure of the slit diaphragm but is also involved in cell signalling. Both nephrin and CD2AP are known to interact with the p85 regulatory subunit of PI-3 kinase to stimulate PI-3 kinase-dependent Akt signalling in podocytes [29]. Akt activation mediates the subsequent phosphorylation and inactivation of several target proteins in podocytes including the pro-apoptotic protein Bad thereby protecting podocytes against detachment-induced cell death [40]. As well as changes in nephrin and CD2AP, we also observed an up-regulation of the plasma membrane protein ezrin and podocalyxin-like protein. Ezrin has also been reported to protect LLC-PK1 cells from apoptosis by activating the PI-3 kinase/Akt pathway [30], while podocalyxin has been shown to exhibit anti-apoptotic properties in malignant cells, probably via its interaction with ezrin [41]. That apoptosis is suppressed by CRP was confirmed by the observed reduction of caspase-3 activity and the increased expression of Bel-2 [31]. Bel-2 is known to have a protective anti-apoptotic role, as down-regulation of this protein is closely associated with the development of progressive
glomerular injury. Low Bcl-2 expression levels are also a clinical prognostic indicator of poor renal outcome patients with IgA nephropathy [42].

In our study, the observed raised levels of caspase-3 activity in control cells was almost certainly a function of the serum-free cell culture conditions that were necessary to avoid the confounding effects of CRP in the serum. The effects of serum-free culture conditions on podocytes have previously been described [43, 44] and suggest that podocytes cultured in serum-free conditions inherently express a pro-apoptotic phenotype.

Treatment of podocytes with the PI-3 kinase inhibitor LY reversed the suppressive effect of CRP on caspase-3 activity as determined by the fluorometric caspase-3 activity assay. This suggests that the CRP-induced decrease in caspase-3 activity is mediated via the PI-3 kinase pathway. However, why LY reduced caspase-3 activity in control cells, when it increased it in CRP-treated cells is not known at this stage. It is possible that in the absence of an active PI-3 kinase pathway to inhibit, LY may have other secondary effects, which lead to the observed decrease in caspase-3 activity. Also puzzling is the fact that despite increasing caspase-3 activity

\[ \text{Fig. 12. Dose response of CRP. (A) Western blot showing effects of CRP at concentrations of 0.1, 0.5, 1.0, 10 and 50 \mu g/mL on podocyte caspase-3 expression. Representative blot is shown. Histogram shows densitometry data of the 17/19-kD subunit from three to eleven experiments (**P < 0.03 versus med, * P < 0.001 versus med). (B) Western blot showing effect of CRP concentrations on nephrin expression. A representative blot is shown. Histogram represents data from four experiments.} \]
in CRP-treated cells, LY unexpectedly appeared to reduce the intensity of the 24- and 17/19-kD subunits (as seen by western blotting). Moreover, this reduction was most marked in the control cells (no CRP). As the PI-3 kinase–Akt signalling pathway acts as a survival (anti-apoptotic) pathway, it is surprising to observe a reduction in active caspase-3 subunits in the presence of a PI-3 kinase inhibitor (which should enhance apoptosis). It is possible that the small 12-kD subunit, which was increased on LY treatment, may have inherent caspase-3 activity. However, in control cells the 12-kD subunit activity in the absence of the larger subunits results in an overall decrease in caspase-3 activity as detected by the fluorimetric assay. This phenomenon clearly requires further investigation but is beyond the scope of the current study.

Akt phosphorylation, although generally up-regulated by CRP (as seen in over 20 assays), was not consistently observed. A counterintuitive down-regulation of Akt phosphorylation in the face of decreased apoptosis has been previously observed by Foster et al. [43] who found that vascular endothelial growth factor was able to suppress apoptosis in serum-starved podocytes while concomitantly reducing AKT phosphorylation. Although we have demonstrated that apoptosis is down-regulated by treatment with CRP, this is the opposite of what occurs in endothelial cells where apoptosis is increased [45] and is one of the mechanisms thought to be involved in CRP’s induction of cardiovascular disease.

Evidence for a relationship between CRP and cell survival has already previously been demonstrated. In cancer cells for example, CRP has been shown to bind Fcγ receptors, activate PI-3 kinase/Akt and inhibit caspase cascade activation induced by chemotherapy drugs [46]. However, while CRP-mediated podocyte survival is desirable, CRP-induced survival of malignant cells is not.

A study by Pepsy group found that of 468 samples of serum from normal adult individuals, 90% had levels <3 µg/mL and 99% had levels <10 µg/mL [47]. Moreover, joint guidelines from the Centres for Disease Control and Prevention and the American Heart Association have set guidelines for the use of CRP levels as an indicator of cardiovascular risk at <1.0 µg/mL as low, 1–3 µg/mL as moderate and >3 µg/mL as high risk [48]. To what extent the culture conditions of our study with the two CRP concentra-

References


Fig. 13. Effect of CRP on podocyte morphology. Cells grown on perma-

nox slides were exposed to increasing concentration of CRP for 24 h. The podocyte actin cytoskeleton was stained with FITC-phalloidin. Representative photomicrographs from three experiments are presented (×400 magnification).


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