Prednisolone inhibits hyperosmolarity-induced expression of MCP-1 via NF-κB in peritoneal mesothelial cells

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The mechanism of peritoneal fibrosis in patients on continuous ambulatory peritoneal dialysis (CAPD) is poorly elucidated. We investigated the cellular mechanism of high-glucose-induced expression of monocyte chemoattractant protein-1 (MCP-1), which is important in recruiting monocytes into the peritoneum and progression of peritoneal fibrosis, and examined the inhibitory mechanism of glucocorticoids. Rat peritoneal mesothelial cells were cultured in high-glucose-containing medium and then analyzed for phosphorylation levels of p42/44 and p38 mitogen-activated protein (MAP) kinases (MAPK), MAPK or extracellular signal-regulated kinase kinase (MEK)1/2, c-Jun N-terminal kinase (JNK)1/2, and protein kinase C (PKC) by Western blotting. Expression of MCP-1 was examined by reverse transcription-polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. DNA-binding activity of nuclear factor (NF)-κB was measured by electrophoretic mobility shift assay. High glucose increased MCP-1 mRNA and MCP-1 protein expression. Although glucose increased phosphorylation of MEK1/2, p42/44 MAPK, p38 MAPK, JNK1/2, and PKC, and DNA-binding activity of NF-κB, its effect on MCP-1 expression was suppressed only by PKC and NF-κB inhibitors. Mannitol caused a similar increase in PKC and NF-κB activation and MCP-1 synthesis. Prednisolone increased I-κB-α expression and inhibited glucose/mannitol-induced NF-κB DNA binding and MCP-1 expression without affecting PKC phosphorylation. The inhibitory effects of prednisolone on MCP-1 expression were reversed by mifepristone, a glucocorticoid receptor antagonist. Our results indicate that glucose induces MCP-1 mainly through hyperosmolarity by activating PKC and its downstream NF-κB, and that such effect was inhibited by prednisolone, suggesting the efficacy of prednisolone in preventing peritoneal fibrosis in patients on CAPD.

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Progressive peritoneal fibrosis has been observed in patients on long-term continuous ambulatory peritoneal dialysis (CAPD). Morphological studies of the peritoneal membrane in patients chronically exposed to dialysis solutions have shown loss of mesothelial cells and replacement of the membrane by collagen-based fibrous tissue.[1,2] Furthermore, some patients develop sclerosing encapsulating peritonitis, a condition associated with high mortality.[3] Therefore, there is a pressing need for a peritoneal dialysate that is less harmful to the peritoneal membrane and has fewer inflammatory and fibrogenetic effects.

Abnormal remodeling of the extracellular matrix results from a complex process that involves increased expression of growth factors and cytokines, which lead to quantitative and constitutional changes in extracellular matrix components. The peritoneal dialysate, containing non-physiological materials such as hypertonic glucose, glucose degradation products, and lactate-buffered acidic solution, is thought to be toxic and is implicated in the long-term damage of the peritoneal membrane.[2,4] In particular, glucose plays an important role in the pathogenesis of peritoneal fibrosis. High glucose levels induce the expression of various cytokines such as transforming growth factor-β[5] and basic fibroblast growth factor,[6] which in turn lead to extracellular matrix production such as fibronectin in mesothelial cells.[7] In addition, glucose suppresses cell–cell or cell–extracellular matrix adhesions of mesothelial cells followed by impaired regeneration of mesothelial cells.[8,9] Glucose is therefore involved in the initiation and progression of peritoneal damage.

The chemokines monocyte chemoattractant protein-1 (MCP-1) has been reported to have a high degree of specificity as a chemotactic factor for monocytes and macrophages, and plays an important role in the initiation and progression of inflammatory processes in peritoneal

fibrosis.[10] Recent studies have shown that the concentration of MCP-1 is increased in the dialysate effluent using conventional glucose-containing dialysate.[11] In addition, specific antibody blocking studies have demonstrated a direct role for mesothelial cell-derived MCP-1 in mediating leukocyte migration across the mesothelial layer,[12] indicating that MCP-1 might play an important role in the progression of peritoneal damage.

Induction of MCP-1 gene expression is mediated through multiple intracellular signal pathways such as p42/44 and p38 mitogen-activated protein kinases (MAPK),[13,14] protein kinase C (PKC),[15] focal adhesion kinase,[16] and nuclear factor (NF)-κB[17,18] in glomerular mesangial cells. In mesothelial cells, glucose is known to induce MCP-1 production through several intracellular signaling pathways, such as PKC,[19] and partly by tyrosine kinase/activated protein (AP)-1 pathway.[20] However, the precise intracellular molecular mechanism in mesothelial cells remains to be elucidated.

Corticosteroids, especially prednisolone, are the pre-eminent anti-inflammatory agents widely used to suppress fibrosis such as that characteristic of various types of glomerulonephritis, interstitial pneumonia, and collagen diseases. Activation of glucocorticoid receptor (GR) results in increased or decreased transcription of a number of genes involved in the inflammatory process.[21] Although glucocorticoids are also used for the treatment of peritoneal impairment such as sclerosing encapsulating peritonitis,[22] the molecular mechanisms responsible for this efficacy remain to be defined. In peritoneal mesothelial cells, corticosteroid suppresses glucose-induced basic fibroblast growth factor expression.[6] To our knowledge, however, there has been no investigation regarding the effect of glucocorticoids on MCP-1 expression in peritoneal mesothelial cells.

The present study was designed to determine the following: (1) the potential mechanisms responsible for the initiation of peritoneal fibrosis, (2) the effects of glucose on various intracellular signal pathways including MAPK, PKC, and NF-κB in rat peritoneal mesothelial cells (RPMC), (3) the pathway responsible for MCP-1 expression, and (4) the molecular mechanism of prednisolone inhibition of glucose-induced MCP-1 expression. The results indicate that glucose induces MCP-1 expression through activation of PKC and its downstream NF-κB and that prednisolone inhibits glucose-induced MCP-1 expression through the inhibition of GR-mediated activation of NF-κB by increasing I-κB-α expression.

RESULTS

High glucose concentration-induced MCP-1 mRNA and protein expression

First, we examined the time-dependent effects of high glucose concentrations on the expression of MCP-1 mRNA. RPMC were incubated for indicated times with 140 mM (2.5%) glucose, which is the glucose concentration found in commercial peritoneal dialysates. MCP-1 mRNA was expressed at low levels in serum-starved RPMC in culture medium containing normal glucose concentrations (5.6 mM). However, MCP-1 mRNA expression increased rapidly in cell culture media containing 140 mM glucose, from 1 h with a peak noted at 3 h (484% of control, $P<0.001$) (Figure 1a). The same concentrations of mannitol, a non-metabolizable osmotic compound, also resulted in a similar and significant increase in MCP-1 mRNA expression (445% of control, $P<0.001$) (Figure 1a). We also examined the effect of different concentrations of glucose and mannitol on MCP-1 mRNA expression. After a 3 h incubation of RPMC with the indicated concentrations of glucose or mannitol, MCP-1 mRNA expression levels were similarly increased in both groups in a concentration-dependent manner (Figure 1b).

In addition, we examined MCP-1 protein expression after exposure of RPMC to 140 mM glucose or 140 mM mannitol for the indicated times (Figure 2a). Although MCP-1 protein levels in RPMC extracts did not change up to 24 h (data not shown), those in culture media increased significantly at 6 h after exposure, and remained increased up to 24 h (glucose, 356 pg/ml, $P<0.0001$; mannitol, 359 pg/ml, $P<0.0001$), after MCP-1 mRNA expression reached a peak level at 3 h. We also examined the effects of different concentrations of glucose and mannitol on MCP-1 protein expression. After 24 h incubation of RPMC with the indicated concentrations of glucose or mannitol, MCP-1 protein expression levels were

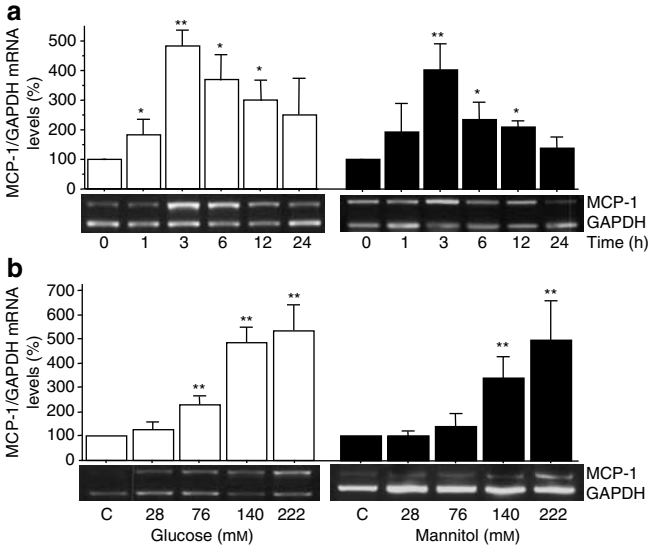

Figure 1 | Expression of MCP-1 mRNA by high glucose and mannitol concentrations. Cultured RPMC were serum starved for 24 h and incubated with 140 mM glucose or 140 mM mannitol for the (**a**) indicated times or with (**b**) indicated concentrations of glucose or mannitol for 3 h. Extracted mRNAs were simultaneously assayed for the expression of MCP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by reverse transcription-polymerase chain reaction (RT-PCR). The amount of PCR product was determined by scanning densitometry and the relative ratio of MCP-1 to GAPDH band density was calculated for each lane. Data are expressed as percentages of the (**a**) amount of time 0 or (**b**) control (5.6 mM glucose or mannitol). Values represent mean ± s.d. ($N=4$). *$P<0.05$; **$P<0.001$ versus control. Open bars, glucose; solid bars, mannitol.

increased in a concentration-dependent manner (Figure 2b). Although the extent of the increase by mannitol was less than that by glucose, there was no significant difference between the effects of glucose and mannitol. We have previously reported that 222 mM glucose exhibited significant cytotoxicity in RPMC; 222 mM glucose resulted in a decrease in G_2/M population, an increase in sub-G_1 population, and an increase in lactate dehydrogenase release,[9] and for this reason we selected glucose at 140 mM concentration in subsequent experiments.

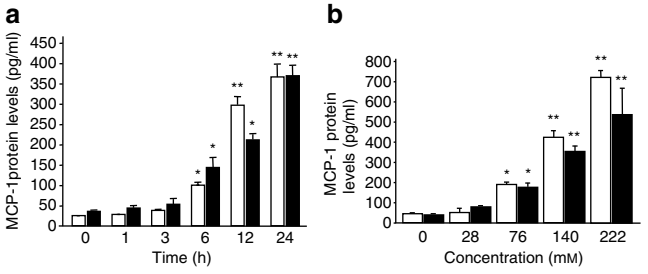

Figure 2 | Expression of MCP-1 protein by high glucose and mannitol concentrations. RPMC were serum starved for 24 h and incubated with 140 mM glucose or 140 mM mannitol for the (**a**) indicated times or with (**b**) indicated concentrations of glucose or mannitol for 24 h. Levels of MCP-1 protein in culture supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA). Values represent mean ± s.d. ($N = 5$). Open bars, glucose; solid bars, mannitol. *$P < 0.001$; **$P < 0.0001$ versus time 0.

Role of PKC on high glucose concentration-induced MCP-1 expression

To explore the mechanism underlying the effects of high glucose on MCP-1 expression, we examined the effects of high glucose on activation of various intracellular signaling molecules. The phosphorylation of MAPK kinase or extracellular signal-regulated kinase kinase (MEK)1/2, p42/44 MAPK, p38 MAPK, c-Jun N-terminal kinase (JNK)1/2, and PKC was examined by Western blot analysis using anti-phospho antibodies (Figures 3a–e). High glucose rapidly increased phosphorylation of each molecule in a time-dependent manner, with a peak noted at 5 min for MEK1/2 (212% of time 0, $P < 0.05$), 30 min for p42/44 MAPK (280%, $P < 0.05$), 5 min for p38 MAPK (163%, $P < 0.05$), 30 min for JNK1/2 (184%, $P < 0.05$), and 15 min for PKC (160%, $P < 0.05$).

Next, we examined the role of each signaling molecule on glucose-induced expression of MCP-1. RPMC were preincubated with or without each inhibitor for MEK, PD98059; JNK, SP600125; p38 MAPK, SB203580; or PKC, calphostin C, for 30 min, and then stimulated with high-glucose-containing

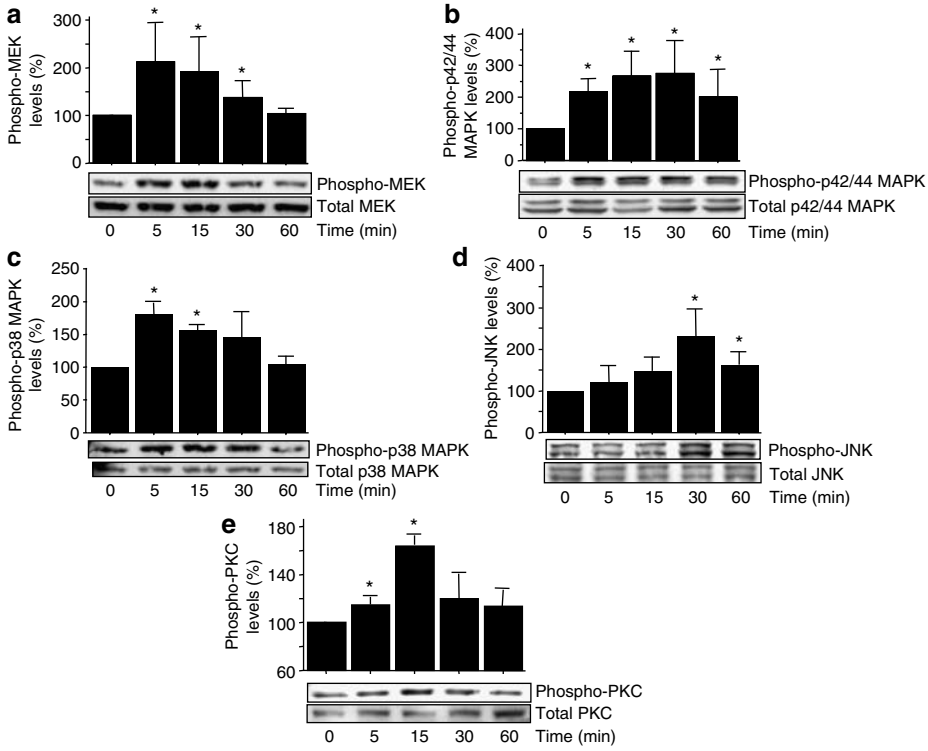

Figure 3 | Effects of high glucose concentrations on the phosphorylation of intracellular signaling molecules. RPMC were serum starved for 24 h and incubated with 140 mM glucose for the indicated times. Equal amounts of cell extracts (40 μg protein) were subjected to electrophoresis and phosphorylation levels of (**a**) MEK1/2, (**b**) p42/44 MAPK, (**c**) p38 MAPK, (**d**) JNK1/2, and (**e**) PKC were assayed by Western blotting using anti-phospho antibodies for each molecule. Relative phosphorylation levels were determined by densitometric analysis and are presented as the relative ratio of phospho-MEK1/2, p42/44 MAPK, p38 MAPK, JNK1/2, and PKC to total MEK1/2, p42/44 MAPK, p38 MAPK, JNK1/2, and PKC, respectively. The value at time 0 was set as 100% and the data are reported as percentages of the amount at time 0. Values represent mean ± s.d. ($N = 5$). *$P < 0.05$ versus time 0.

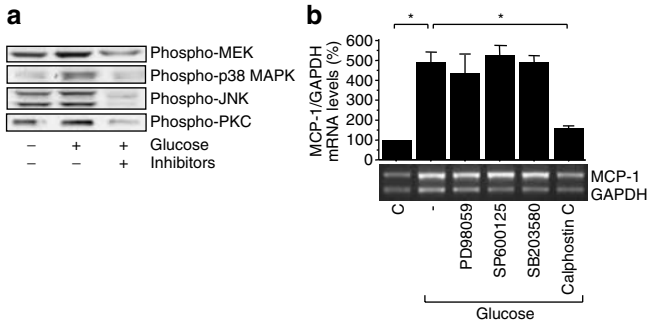

Figure 4 | Effects of various inhibitors on high-glucose-induced MCP-1 mRNA expression. (**a**) RPMC were serum starved for 24 h and pretreated with or without the indicated inhibitors (25 μM PD98059 for MEK, 30 μM SP600125 for JNK, 10 μM SB203580 for p38 MAPK, 0.33 μM calphostin C for PKC) for 30 min before and during incubation with 140 mM glucose. RPMC were incubated with glucose for 5 min for MEK, 5 min for p38 MAPK, 30 min for JNK, or 15 min for PKC. Equal amounts of cell extracts (40 μg protein) were subjected to electrophoresis and phosphorylation levels of MEK1/2, p38MAPK, JNK1/2, and PKC were assayed by Western blotting using anti-phospho antibodies for each molecule. (**b**) RPMC were preincubated with or without the indicated inhibitors for 30 min before incubating with 140 mM glucose for 3 h, and assayed for MCP-1 mRNA expression by RT-PCR. Data represent mean ± s.d. ($N = 4$). *$P < 0.0001$.

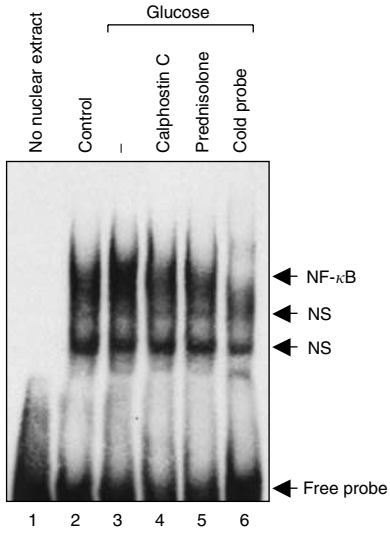

Figure 5 | EMSA of high-glucose-induced DNA-binding activity of NF-κB. RPMC were serum starved for 24 h and nuclear extracts were prepared from cells treated with or without 140 mM glucose for 90 min. Cells were preincubated with or without 0.33 μM calphostin or 1 μM prednisolone 30 min before adding glucose (lanes 4 and 5). Equal amounts (5 μg of protein) of the nuclear extracts were assayed for the ability to bind a biotin-labeled double-stranded NF-κB oligonucleotide. As a specificity control, a 100-fold excess of unlabeled probe was added (lane 6). The positions of NF-κB and free probe are indicated. NS: nonspecific bands.

medium. Each inhibitor completely suppressed glucose-induced activation of MEK1/2, p38 MAPK, JNK1/2, and PKC (Figure 4a). We confirmed the specific inhibitory effect of each inhibitor. PD98059, SP600125, SB203580, and calphostin C specifically inhibited phosphorylation of MEK, p38 MAPK, JNK, and PKC, respectively, without affecting phosphorylation of other molecules (data not shown), indicating that the concentrations used in the present study produce specific inhibition of each of the respective signaling molecules. Glucose-induced MCP-1 mRNA expression was abolished only by calphostin C (increase by glucose was inhibited by 84.9%, $P < 0.0001$), but not by PD98059, SP600125, and SB203580 (Figure 4b), suggesting that PKC is responsible for mediating high-glucose-induced MCP-1 mRNA expression.

Role of NF-κB on high glucose concentration-induced MCP-1 expression

PKC has been reported to activate downstream transcriptional factor, NF-κB, in many cell types. We therefore examined the role of NF-κB on glucose-induced MCP-1 expression. Activation of NF-κB by high glucose was confirmed by electrophoretic mobility shift assay (EMSA), which revealed that incubation of RPMC with high-glucose-containing medium increased DNA-binding activity of NF-κB (155% of control, $P < 0.05$; Figure 5, lane 3). The increase in DNA-binding activity of NF-κB was completely suppressed by PKC inhibitor, calphostin C, indicating that NF-κB locates downstream of PKC in mediating glucose-induced NF-κB activation (118% of control; Figure 5, lane 4). As a specificity control, a 100-fold excess amount of unlabeled cold probe was added as a competitor. The use of this control resulted in complete competition for NF-κB DNA binding (Figure 5, lane 6).

We then examined the effects of NF-κB inhibitors on glucose-induced MCP-1 mRNA expression. To inhibit NF-κB activity, RPMC were pretreated with either protease inhibitor tosyl phenylalanyl chloromethylketone (TPCK), which has been shown to inhibit interleukin (IL)-1-inducible NF-κB, or antioxidant pyrrolidinedithiocarbamate (PDTC), which has been shown to block phorbol ester-induced NF-κB activation.[17] High-glucose-induced expression of MCP-1 mRNA was partially suppressed by TPCK by 51% ($P < 0.05$) and PDTC by 68% ($P < 0.05$), suggesting that NF-κB is involved in mediating high-glucose-induced MCP-1 mRNA expression (Figure 6a). The suppression by PKC and NF-κB inhibitors of glucose-induced MCP-1 mRNA expression (Figures 4b and 6a) confirmed the effects of these inhibitors on MCP-1 protein expression. Thus, MCP-1 protein expression was completely suppressed by the PKC inhibitor, calphostin C, and partially by NF-κB inhibitors, PDTC or TPCK, consistent with the results in MCP-1 mRNA (Figure 6b).

Effects of prednisolone on glucose-induced MCP-1 mRNA and protein expression

We further investigated the effects of prednisolone on glucose-induced MCP-1 expression.[3] We initially confirmed the expression of GR in cultured RPMC by RT-PCR, which showed expression of GR mRNA in RPMC (Figure 7a). We

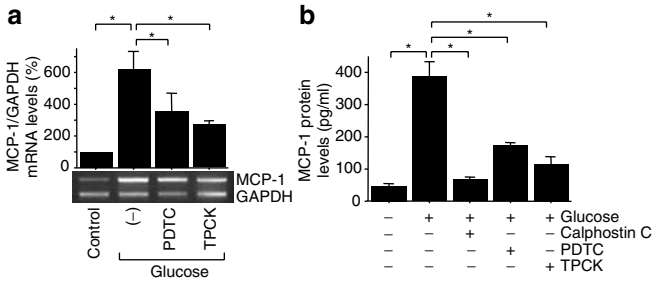

Figure 6 | Effects of NF-κB inhibitors on high-glucose-induced MCP-1 mRNA and protein expression. Serum-starved RPMC were pretreated with or without calphostin C (0.33 μM), PDTC (50 μM), or TPCK (5 μM) for 30 min, and incubated with 140 mM glucose for (**a**) 3 h or (**b**) 24 h. Expression levels of MCP-1 mRNA and protein were assayed by (**a**) RT-PCR and (**b**) ELISA, respectively. Values represent mean ± s.d. ($N = 5$). *$P < 0.05$.

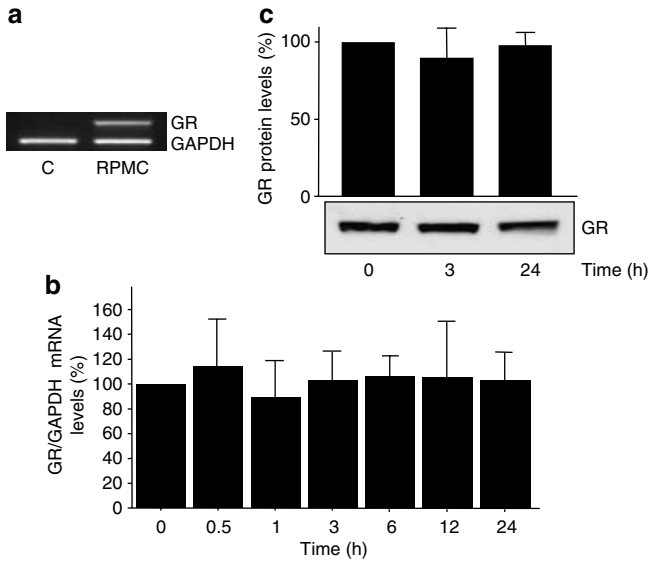

Figure 7 | Expression of GR in RPMC. (**a**) Expression of GR in RPMC was confirmed by RT-PCR using specific primers for GR. Non-reverse-transcribed RNA was used for negative control. Serum-starved RPMC were incubated with 140 mM glucose for the indicated times and expression levels of GR mRNA and protein were analyzed by (**b**) RT-PCR and (**c**) Western blotting, respectively. Values represent mean ± s.d. ($N = 5$).

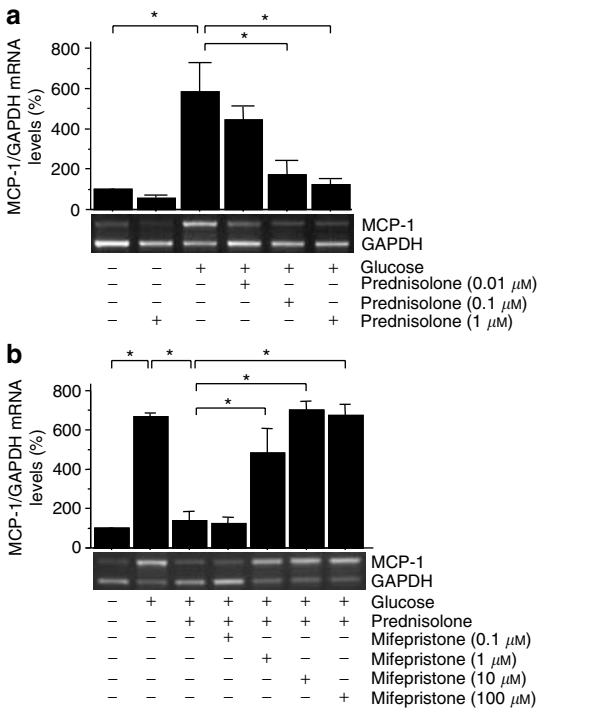

Figure 8 | Effects of prednisolone on high-glucose-induced expression of MCP-1 mRNA. (**a**) Serum-starved RPMC were pretreated with or without indicated concentrations of prednisolone for 30 min before incubation with 140 mM glucose for 3 h. Expression of MCP-1 was assayed by RT-PCR. Data were calculated from relative expression levels of MCP-1 to GAPDH and are expressed as percentages of the amount of cells without glucose and prednisolone. Values represent mean ± s.d. ($N = 5$). *$P < 0.05$. (**b**) Serum-starved RPMC were preincubated with the indicated concentrations of mifepristone for 30 min before adding 1 μM prednisolone and incubation with 140 mM glucose for 3 h. Expression of MCP-1 was assayed by RT-PCR. Data were calculated from relative expression levels of MCP-1 to GAPDH and are expressed as percentages of the amount of cells without glucose, prednisolone, and mifepristone. Values represent mean ± s.d. ($N = 4$). *$P < 0.05$.

also examined the effect of high glucose on the expression level of GR to exclude the possibility that the effects of high glucose might result from altered GR expression levels. High glucose had no effects on GR mRNA and protein levels throughout the experiment up to 24 h (Figures 7b and c). The same concentration of mannitol (140 mM) had no effect on GR protein expression levels (data not shown).

Next, we examined the effect of prednisolone on glucose-induced MCP-1 mRNA expression. Prednisolone completely inhibited high-glucose-induced MCP-1 mRNA expression in a dose-dependent manner (Figure 8a). Suppression levels of the increase were 85.4% in 0.1 μM prednisolone and 95.6% in 1 μM prednisolone. We subsequently examined whether this prednisolone effect on MCP-1 mRNA expression was mediated by prednisolone binding to GR. RPMC were preincubated with the indicated concentrations of mifepristone, a GR antagonist,[23] followed by the addition of 140 mM glucose and 1 μM prednisolone. Mifepristone at 1 μM significantly suppressed prednisolone inhibition of glucose-induced MCP-1 mRNA expression, and more than 10 μM concentrations of mifepristone completely attenuated the effects of prednisolone (Figure 8b). In the absence of glucose, mifepristone had no significant effects on MCP-1 mRNA (data not shown) and protein expression (Figure 9). Mifepristone also reduced the inhibitory effect of prednisolone on high-glucose-induced MCP-1 protein excretion (Figure 9), indicating that the effects of prednisolone on MCP-1 expression were mediated by prednisolone binding to GR.

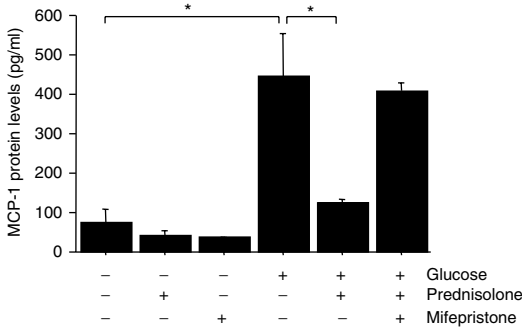

Figure 9 | Effects of prednisolone on high-glucose-induced MCP-1 protein secretion. Serum-starved RPMC were preincubated with or without 10 μM mifepristone for 30 min before incubation with or without 1 μM prednisolone for 30 min followed by 140 mM glucose for 24 h. The levels of MCP-1 protein in the culture medium were assayed by ELISA. Values represent mean ± s.d. ($N = 7$). *$P < 0.05$.

Mechanism of prednisolone inhibition of glucose-induced MCP-1 expression

We further examined the molecular mechanism of the inhibitory effects of prednisolone on glucose-induced MCP-1 expression after binding of prednisolone to GR. Prednisolone completely suppressed glucose-induced DNA-binding activity of NF-κB as effectively as a PKC inhibitor, calphostin C (Figure 5, lanes 4 and 5). However, prednisolone had no effect on high-glucose-induced phosphorylation of PKC (Figure 10a), suggesting that the effects of prednisolone on glucose-induced MCP-1 expression were mediated by NF-κB, downstream of PKC.

Glucocorticoids are known to inhibit NF-κB activation at different levels. We therefore examined the mechanism of prednisolone action on NF-κB. It has been postulated that steroids inhibit NF-κB activation by increasing the transcription of I-κB-α, an endogenous inhibitor of NF-κB.[24,25] We initially examined the effect of prednisolone on the

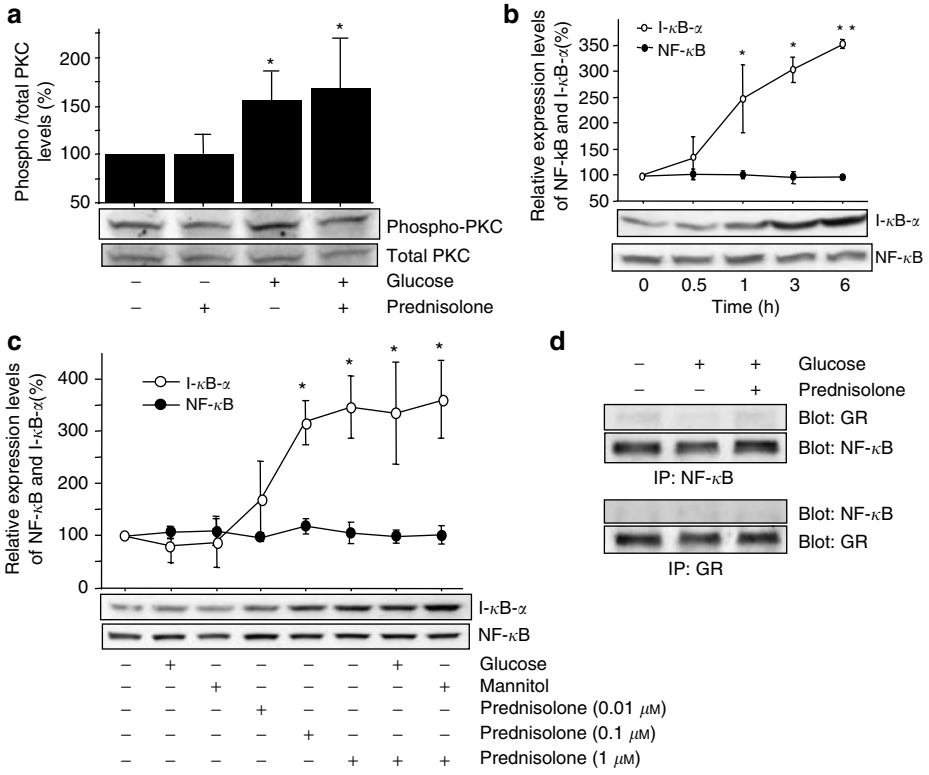

Figure 10 | The mechanism of prednisolone inhibition of glucose-induced MCP-1 expression. (**a**) Effect of prednisolone on high-glucose-induced PKC phosphorylation. Serum-starved RPMC were preincubated with or without 1 μM prednisolone for 30 min and incubated with or without 140 mM glucose for 15 min. Phosphorylation levels of PKC were assayed by Western blotting using a specific antibody against phospho-PKC. Phosphorylation levels were expressed relative to the value at time 0. Values represent mean ± s.d. ($N = 5$). *$P < 0.01$. (**b**) Effects of prednisolone on NF-κB and I-κB-α protein expressions. Serum-starved RPMC were incubated with 1 μM prednisolone for the indicated times. Expression levels of NF-κB and I-κB-α were assayed by Western blotting. Expression levels are expressed relative to the value at time 0. Values represent mean ± s.d. ($N = 4$). *$P < 0.05$; **$P < 0.01$. (**c**) Dose response of prednisolone-induced I-κB-α protein expression and effects of glucose and mannitol on prednisolone-induced I-κB-α protein expression. Serum-starved RPMC were preincubated with or without the indicated concentrations of prednisolone for 30 min and incubated with glucose or mannitol for 6 h. Expression levels of NF-κB and I-κB-α were assayed by Western blotting. Expression levels are expressed relative to the value without glucose, mannitol, or prednisolone. Values represent mean ± s.d. ($N = 4$). *$P < 0.05$. (**d**) Effects of prednisolone on association of GR and NF-κB. Serum-starved RPMC were preincubated with or without 1 μM prednisolone for 30 min and incubated with or without 140 mM glucose for 90 min. NF-κB was immunoprecipitated (IP) and immunoblotted for NF-κB and GR (upper panel). Alternatively, GR was immunoprecipitated and immunoblotted for NF-κB and GR (lower panel).

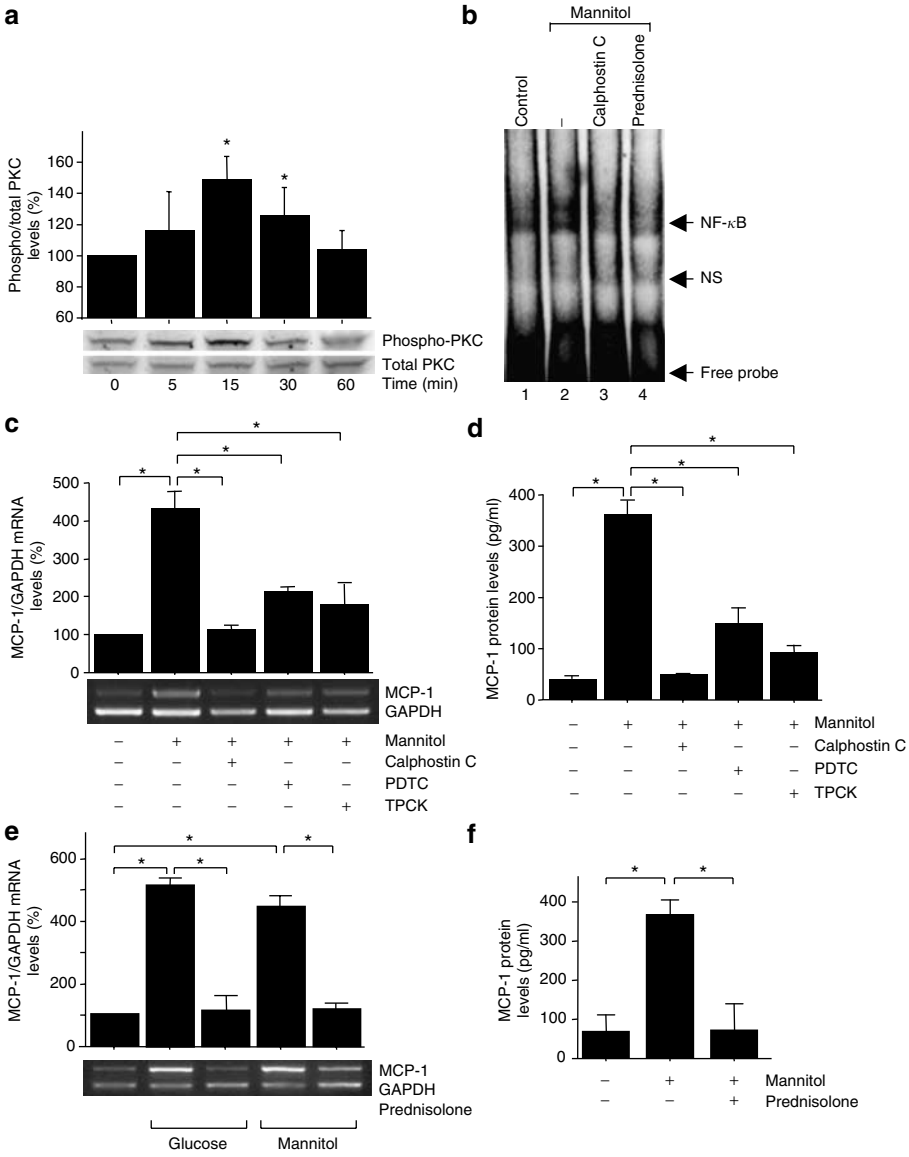

Figure 11 | Effects of hyperosmolarity on MCP-1 expression. (**a**) Effects of mannitol on PKC phosphorylation. RPMC were serum starved for 24 h and incubated with 140 mM mannitol for indicated times. Phosphorylation levels of PKC were assayed by Western blotting. Values represent mean ± s.d. ($N = 5$). *$P < 0.05$ versus time 0. (**b**) EMSA of mannitol-induced DNA-binding activity of NF-κB. RPMC were serum starved for 24 h and nuclear extracts were prepared from cells treated with or without 140 mM glucose for 90 min. Cells were preincubated with or without 0.33 μM calphostin or 1 μM prednisolone 30 min before adding mannitol (lanes 3 and 4). Equal amounts (5 μg of protein) of nuclear extracts were assayed for binding a biotin-labeled double-stranded NF-κB oligonucleotide. The positions of NF-κB and free probe are indicated. NS: nonspecific bands. (**c**, **d**) Effects of PKC and NF-κB inhibitors on mannitol-induced MCP-1 mRNA and protein expression. Serum-starved RPMC were pretreated with or without calphostin C (0.33 μM), PDTC (50 μM), or TPCK (5 μM) for 30 min, and incubated with 140 mM mannitol for 3 h (**c**) or 24 h (**d**). Expression levels of MCP-1 mRNA and protein were assayed by RT-PCR and ELISA, respectively. Values represent mean ± s.d. ($N = 5$). *$P < 0.05$. (**e**, **f**) Effects of prednisolone on glucose- and mannitol-induced MCP-1 mRNA and protein. Serum-starved RPMC were preincubated with or without 1 μM prednisolone for 30 min and incubated with or without 140 mM glucose or mannitol for (**e**) 3 h or (**f**) 24 h. Expression levels of MCP-1 mRNA and protein were assayed by RT-PCR or ELISA, respectively. Values represent mean ± s.d. ($N = 4$). *$P < 0.05$.

expression of I-κB-α by Western blotting. The results showed that prednisolone upregulated I-κB-α in a time-dependent manner, but not the expression of NF-κB for up to 6 h (Figure 10b). Prednisolone also increased I-κB protein expression in a dose-dependent manner, without affecting NF-κB expression (Figure 10c). Glucose and mannitol slightly reduced, although not significantly, both I-κB and NF-κB expression. Furthermore, prednisolone upregulated I-κB even in the presence of high glucose and mannitol concentrations. The activated GR is also reported to bind to the p65 subunit of NF-κB, and thus interferes with the binding of NF-κB to DNA and inhibits NF-κB-driven gene expression.[26] We therefore examined the binding of p65 NF-κB to GR by co-immunoprecipitation. Our data showed a

weak association of these molecules and no increase in the binding by prednisolone treatment (Figure 10d), suggesting that the effects of prednisolone were mediated by increasing the transcription of I-κB-α, not by increasing the binding of p65 NF-κB to GR in RPMC.

Effects of high osmolarity on MCP-1 expression levels

Finally, we examined the effects of high osmolarity on MCP-1 expression. Mannitol at concentration similar to that of glucose produced glucose-equivalent increase in PKC phosphorylation levels, with a peak noted at 15 min (Figure 11a). Mannitol also increased the DNA-binding activity of NF-κB (Figure 11b). The latter effect was completely suppressed by PKC inhibitor, calphostin C, and prednisolone. Mannitol also increased MCP-1 mRNA and protein expression levels (Figures 11c and d); the effects were completely abolished by calphostin C and partially by PDTC or TPCK as seen in glucose-induced MCP-1 mRNA and protein expression, suggesting that PKC is essential and NF-kB is partially involved in mediating mannitol-induced MCP-1 expression. Likewise, prednisolone completely suppressed mannitol-induced MCP-1 mRNA and protein expression (Figures 11e and f), suggesting that high osmolarity contributes to the regulation of MCP-1 expression, and that prednisolone has similar inhibitory effects on glucose- and mannitol-induced MCP-1 expression.

DISCUSSION

Glucocorticoid treatment prevents the progression of peritoneal fibrosis and peritoneum adhesion;[22] however, there have been few investigations into the molecular mechanisms of glucocorticoids in peritoneal fibrosis. It has been reported that prednisolone suppresses glucose-induced basic fibroblast growth factor and laminin expression in peritoneal mesothelial cells.[6,27] To our knowledge, there has been no investigation regarding the effects of glucocorticoids on intracellular signal pathways and MCP-1 expression in peritoneal mesothelial cells. Our study provides the first evidence that prednisolone inhibits high-glucose-induced MCP-1 expression by inhibiting the action of NF-κB in peritoneal mesothelial cells. These results suggest that prednisolone might be effective in preventing fibrosis in patients receiving CAPD.

In the present study, we showed that glucose-induced MCP-1 expression is mediated by PKC and NF-κB. Platelet-derived growth factor induces MCP-1 expression via p42/44 MAPK, JNK, and p38 MAPK, suggesting that MAPK families also play important roles in MCP-1 expression.[13,28] However, Ha *et al.*[18] reported that glucose induces MCP-1 expression via PKC and its downstream NF-κB pathway independent of MAPK pathways, consistent with our results. Inhibition of p42/44 MAPK, JNK, and p38 MAPK fails to suppress Toll-like receptor-4-mediated MCP-1 expression.[29] Furthermore, IL-1β-induced MCP-1 expression via p38 MAPK is not mediated by NF-κB,[14] suggesting that p38 MAPK–CREB and PKC–NF-κB independently regulate MCP-1 expression. These reports indicate the involvement of several pathways, in addition to MAPK, in the induction of MCP-1 expression.

NF-κB is a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors.[30] NF-κB dimers are sequestered in the cytosol of unstimulated cells via non-covalent interactions with inhibitor proteins, called I-κBs. Degradation of I-κB proteins allows NF-κB to move into the nucleus after phosphorylation of I-κB. The mechanism of NF-κB suppression by glucocorticoids has been extensively reported. There are several potential levels of interaction between steroids and NF-κB. The activated GR binds to glucocorticoid-responsive elements in the promoter of the I-κB-α gene and induces the expression of I-κB-α.[24,25] This scenario is consistent with our data showing that prednisolone enhanced the expression of I-κB-α (Figure 10b). Thus, newly synthesized I-κB-α can sequester NF-κB, thereby terminating NF-κB-dependent transcriptional activation. Alternatively, activated GR directly binds to NF-κB to prevent NF-κB binding to DNA.[26] However, this hypothesis is unlikely to account for the results observed in this study, because protein interaction between GR and NF-κB was weak and was not increased by prednisolone (Figure 10c). Transactivation by NF-κB depends on the co-activators, cAMP-responsive element-binding protein-binding protein and steroid receptor co-activator-1, for maximal activity.[31] GR-mediated repression of NF-κB-dependent gene expression results from competition for a limited amount of these transcriptional co-activators between NF-κB and GR. Glucocorticoids also repress NF-κB-driven genes by disturbing the interaction of NF-κB with the basal transcription machinery, irrespective of co-activator levels.[32] However, our data do not support these potential mechanisms; these mechanisms interfere with the transactivating potential of NF-κB without affecting DNA binding, whereas prednisolone inhibited the DNA-binding levels of NF-κB in the present study (Figure 5). Thus, our results suggest that high-glucose-induced activation of NF-κB is likely to be inhibited by prednisolone-induced expression of the inhibitor, I-κB-α.

Expression of GR has been investigated in monocytes,[33] mesangial cells,[34] macrophages,[35] and human umbilical vein endothelial cells.[36] In this study, we showed that RPMC express GR as well as human peritoneal mesothelial cell,[6] and that the effect of prednisolone on MCP-1 expression was reversed by a GR antagonist. These results suggest that the effects of prednisolone are mediated by prednisolone binding to GR. We also showed that glucose had no effect on GR expression levels, suggesting that the inhibitory effects of prednisolone on glucose-induced MCP-1 expression were not due to altered expression levels of GR by high glucose levels.

NF-κB can be activated by exposure of cells to high glucose levels, lipopolysaccharides, growth factors, inflammatory cytokines such as tumor necrosis factor or IL-1, viral infection or expression of certain viral gene products, UV irradiation, B- or T-cell activation, and by other physiological and non-physiological stimuli.[18,30] NF-κB regulates the

expression of numerous proteins involved in inflammation upon stimulation, which include cytokines (IL-6), chemokines (IL-8, MCP-1, and regulated upon activation, normal T cell expressed and secreted (RANTES)), and cell adhesion molecules (intercellular adhesion molecule-1 and vascular adhesion molecule-1).[37] The signaling mechanisms mediating these effects include p42/44 MAPK, p38 MAPK, and PKC.[38,39] In the present study, glucose-induced expression of MCP-1 was mediated by PKC and its downstream NF-κB. Interestingly, glucose-induced MCP-1 expression was partially suppressed by NF-κB inhibitors, TPCK by 51% and PDTC by 68%, whereas a PKC inhibitor completely inhibited MCP-1 expression, suggesting that there are other downstream pathways besides NF-κB such as AP-1. These results are consistent with previous reports that high glucose induces MCP-1 expression partly via the AP-1 pathway in human mesothelial cells,[20] and that cytokine induction of MCP-1 expression in human endothelial cells depends on the cooperative action of NF-κB and AP-1.[40] AP-1 is known to be downregulated by glucocorticoid-activated GR;[41] therefore, it might be an additional target of prednisolone inhibition of glucose-induced MCP-1 expression.

MCP-1 is implicated in the progression of various human and experimental glomerulonephritis[42] and diabetic nephropathy[43] as well as peritoneal damage in patients on CAPD[10,12,44] by functioning as a major chemotactic factor for monocytes/macrophages. Our results indicating that prednisolone inhibits glucose-induced expression of MCP-1 suggest the potential efficacy of glucocorticoids in the prevention of peritoneal fibrosis, because mesothelial cell-derived chemokines such as MCP-1 might contribute to the intraperitoneal recruitment of leukocytes in the initiation of peritoneal inflammation that eventually leads to fibrosis.[12] However, our data do not support a role for MCP-1 and glucocorticoids in the later phase of fibrosis, because changes in peritoneal morphology progressively develop during the long-term course of CAPD therapy.[1] In assessing the clinical application of glucocorticoids, it would be important to determine the role of MCP-1 in patients on long-term CAPD use.

So far, the effects of mannitol on MCP-1 expression remain controversial. Haslinger *et al.*[19] reported that high glucose induced MCP-1 expression whereas mannitol did not influence MCP-1 expression levels in human mesothelial cells. However, Wong *et al.*[45] reported that glucose-mediated induction of MCP-1 in human mesothelial cells is osmolarity dependent. In the present study, mannitol had similar effects on MCP-1 expression, suggesting that not only high glucose concentration but also hyperosmolarity *per se* might play a role in these effects. The use of the iso-osmolar glucose polymer icodextrin, which is clinically used as an alternative to glucose in peritoneal dialysis solutions, would be an attractive way to avoid the effects of high osmolarity on MCP-1 expression.

In conclusion, our results suggest that hyperosmolar dialysates containing high glucose concentrations might be involved in the initiation of peritoneal fibrosis, by inducing MCP-1. As NF-κB activates many immunoregulatory genes including MCP-1,[38] specific inhibition of NF-κB activation by glucocorticoids might be an attractive strategy for therapeutic intervention of peritoneal fibrosis in patients undergoing long-term CAPD therapy.

MATERIALS AND METHODS

Materials

Polyclonal antibodies for p42/44 MAPK, phospho- MEK1/2, MEK1/2, and phospho-pan PKC and monoclonal antibody for phospho-p42/44 MAPK were purchased from New England Biolabs (Beverly, MA, USA). The monoclonal antibody for pan PKC was obtained from Sigma (St Louis, MO, USA). Polyclonal antibodies for phospho-p38 MAPK and phospho-c-Jun N-terminal kinase (JNK)1/2 were purchased from BioSource International (Camarillo, CA, USA). MEK inhibitor, PD98059, p38 MAPK inhibitor, SB203580, JNK inhibitor, SP600125, and PKC inhibitor, calphostin C, were purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). To inhibit NF-κB activation, antioxidant PDTC (Sigma) or protease inhibitor TPCK (Sigma) was used. Prednisolone and a GR antagonist, mifepristone, were obtained from Sigma. Anti-p65 NF-κB, I-κB-α, and GR antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). D-Glucose and D-mannitol were purchased from Nacalai (Tokyo, Japan) and used for cell culture immediately after 0.2 μm filter sterilization to avoid the effects of glucose degradation products.

Cell culture

RPMC were obtained and identified as described previously.[9] RPMC were maintained in Dulbecco's modified Eagle's medium containing 5.6 mM glucose, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°C. To synchronize the cells in quiescence, RPMC were maintained in Dulbecco's modified Eagle's medium containing 0.5% fetal bovine serum for 24 h before the experiments. RPMC were used between passages 3 and 6.

Western blotting, immunoprecipitation, and ELISA

Phosphorylation of MEK1/2, p42/44 MAPK, p38 MAPK, JNK1/2, and PKC was analyzed by Western blotting using antibodies recognizing phosphorylated forms of each signal molecule.[46–48] The expression levels of I-κB-α and p65 NF-κB were analyzed by Western blotting using anti-I-κB-α (1:1000) or p65 NF-κB (1:1000) antibodies, respectively. Immunoprecipitation was performed using anti-p65 NF-κB (5 μg/ml) or GR (5 μg/ml) antibodies using RIPA buffer.[46] Immunoprecipitates were analyzed by Western blotting with anti-p65 NF-κB (1:1000) or GR (1:1000) antibodies. MCP-1 concentrations in the culture supernatants and cell extracts of RPMC were measured by using an ELISA kit (BioSource International).[13]

RT-PCR

RT-PCR was performed as described previously using the QuickPrep mRNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) and a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech).[13,49,50] Expression of MCP-1, GAPDH, and GR mRNAs was determined by RT-PCR using specific primers as described.[6,13] PCRs for MCP-1 and GAPDH were simultaneously performed for 24 cycles in the same microtubes under the following conditions: 1 min at 95°C, 45 s at 60°C, and 45 s at 72°C. PCR for

GR was performed for 24 cycles: 1 min at 95°C, 45 s at 60°C, and 45 s at 72°C. Primer sets for MCP-1, GAPDH, and GR generated 595, 308, and 536 bp products, respectively.

Extraction of nuclear protein and EMSA

Nuclear extracts were prepared by using a nuclear extraction kit (Sigma). EMSA was performed by using an EMSA Gel-Shift kit (Panomics, Redwood City, CA, USA). Equal amounts (5 μg each of nuclear extract) were incubated with biotin-labeled double-stranded NF-κB oligonucleotides (Panomics), and nucleoprotein–oligonucleotide complexes were resolved by electrophoresis in 6% non-denaturing polyacrylamide gels.

Statistical analysis

Data are expressed as mean ± s.d. Differences between groups were examined for statistical significance using one-way analysis of variance or the Student's *t*-test. A *P*-value less than 0.05 was considered to represent a statistically significant difference.

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