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Hypertonicity-Induced Expression of Monocyte Chemoattractant Protein-1 through a Novel cis-Acting Element and MAPK Signaling Pathways

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MCP1 is upregulated by various stimuli, including LPS, high glucose, and hyperosmolality. However, the molecular mechanisms of transcriptional regulation of the MCP1 gene under hyperosmolar conditions are poorly understood. Treatment of NRK52E cells with NaCl or mannitol resulted in significant elevation of MCP1 mRNA and protein in a time- and dose-dependent manner. Treatment with a p38MAPK inhibitor (SB203580), an ERK inhibitor (PD98059), or an MEK inhibitor (U0126), suppressed the increase in MCP1 expression caused by hypertonic NaCl, whereas a JNK inhibitor (SP600125) and an API inhibitor (curcumin) failed to attenuate MCP1 mRNA expression by NaCl. In the 5'-flanking region of the MCP1 gene, there is a sequence motif similar to the consensus TonE/ORE as well as the consensus C/E binding protein (BP), NF-κB, and AP1/Sp1 sites. Luciferase activity in cells transfected with reporter constructs containing a putative TonE/ORE element (MCP1-TonE/ORE) enhanced reporter gene expression under hypertonic stress. Results of electrophoretic gel mobility shift assay showed a slow migration of the MCP1-TonE/ORE probe, representing the binding of TonEBP/OREBP/NFAT5 to this enhancer element. These results indicate that the 5'-flanking region of MCP1 contains a hypertonicity-sensitive cis-acting element, MCP1-TonE/ORE, as a novel element in the MCP1 gene. Furthermore, p38MAPK and MEK–ERK pathways appear to be, at least in part, involved in hypertonic stress-mediated regulation of MCP1 expression through the MCP1-TonE/ORE. The Journal of Immunology, 2010, 184: 5253–5262.
hypersmolality. Recent studies on intracellular signaling pathways responsive to hypertonicity have suggested that MAPKs pathways play a role in the cellular mechanisms underlying the regulation of gene expression in response to hypertonicity (21–24).

Taken together, we hypothesized that MCP1 expression induced by hypersmolality may be regulated through a hypertonicity response element, such as TonE/ORE or NF-kB, in the MCP1 gene. Interestingly, in a homologous search of the MCP1 gene sequence and TonE/ORE, there is a sequence motif similar to TonE/ORE in the proximal region of the 5′-flanking region of the MCP1 gene. Therefore, to investigate the molecular basis of transcriptional regulation of MCP1 in response to hypertonicity, we sought to identify a functionally specific enhancer element in response to hypertonic stress in the MCP1 gene, and we examined the intracellular signaling pathways participating in the regulation of MCP1 expression. In this study, we report that in NRK52E cells, MCP1 is upregulated through a novel hypertonicity-sensitive cis-acting element localized in the proximal region of the MCP1 gene, and p38 MAPK and MEK–ERK are, at least in part, implicated in cellular signaling pathways leading to significant expression of MCP1 mRNA and protein under NaCl- and mannitol-induced hypersmolality.

Materials and Methods

RT-PCR analysis

Normal rat kidney (NRK52E) cells (ATCC#: CRL-1571) were exposed to hypertonic stress by addition of NaCl (100 mM) or mannitol (200 mM). At 3, 6, 9, 12 and 24 h after treatment, total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA), in accordance with the manufacturer’s instructions. In addition, NRK52E cells were exposed to glucose (100 mM) for 3 d, or 10 mg/mg albumin or 1 μg/μl LPS for 24 h. Furthermore, NaCl (50, 100 or 150 mM), mannitol (100, 200, or 300 mM), glycerol (200 mM) or urea (200 mM) were also applied to cells for 3 h. RT-PCR was performed using the following primers: for MCP1, sense, 5′-GTGTTCA-CAGTGCTGCTGT-3′; and antisense, 5′-CTACAGAGAATGCTTGA-GTG-3′; and for GAPDH, sense, 5′-AATGCATCCTGCACCACCAA-3′, antisense, 5′-ATAGCCATATGCTCAGGATC-3′.

Inhibition of protein synthesis, PKC, NF-κB, NADPH oxidase and MAPKs, and iron chelation

NRK52E cells were pretreated with 10 μg/ml cycloheximide (CHX; Sigma-Aldrich, St. Louis, MO), a protein synthesis inhibitor; 500 nM calpentin C (Calbiochem, San Diego, CA), a protein kinase C (PKC) inhibitor; 20 μM pyrrolidine dithiocarbamate (PDTC; Sigma-Aldrich, St. Louis, MO), an NF-κB inhibitor; 10 μM dithiothreitol (DTT; Sigma-Aldrich), an NADPH oxidase inhibitor; or 100 μM deferoxamine (DFO; Sigma-Aldrich), an iron chelator. At 1 h after treatment with inhibitors, cells were exposed to NaCl (100 mM, 3 h), fructose (500 mM) or glucose (200 mg/ml cycloheximide), cells were pretreated with SB203580 (5, 10, or 20 μM; Calbiochem), a p38 MAPK inhibitor; PD98059 (5, 10 or 20 μM, Calbiochem), an ERK inhibitor, U0126 (5, 10, or 20 μM; Calbiochem), an MEK inhibitor; SP600125 (10, 20, or 30 μM; Calbiochem), a JNK inhibitor; or curcumin (5 or 15 μM; Sigma-Aldrich), an AP1 inhibitor, at the indicated doses for 1 h, followed by exposure to NaCl (100 mM, 3 h). RT-PCR analysis was performed as described above.

Western blot analysis

For analysis of MCP1 expression induced by hypersmolality, cells were exposed to NaCl (100 mM) or mannitol (200 mM) for 6, 9, 12, 15, and 24 h. In addition, NaCl (50, 100, 150, or 200 mM) or mannitol (200, 300, or 400 mM) was applied to cells for 12 h. For analysis of changes in MAPK activity in response to hypertonicity, cells were treated with NaCl (100 mM) for 15, 30, 60, and 180 min. Moreover, cells were pretreated with MAPK inhibitors for 1 h, followed by exposure to NaCl (100 mM, 30 min). Pelleter cells were transfected with luciferase reporter plasmid containing 2.5-kb fragment of the MCP1 gene (GenBank accession no. AF079313), the promoter region of the MCP1 gene (−939 to +59) was amplified using rat genomic DNA prepared from NRK52E cells and the following primers: sense, 5′-TCTTCTCCTAGTCTGTTG-3′ (−939 to −920) and antisense, 5′-AGAGATCTGCTCCAGTGAG-3′ (+40 to +59). The following primer set: for D1, 5′-CAGGGAATCTTGGAGCAAT-3′ (−759 to −730), for D2, 5′-CCAGGTT-GGAATTGACAA-3′ (−559 to −540), for D3, 5′-AGTATCTTCTCTCC-TTAGGA-3′ (−359 to −340), for D4, 5′-ATCTTCTGCTCAATGTCG-3′ (−219 to −200), for D5, 5′-ATCCGGCGGTCTCCTCTTCT-3′ (−179 to −160).

Luciferase reporter gene construction

On the basis of sequence data of the rat MCP1 gene (GenBank accession no. AF079313), the promoter region of the MCP1 gene (−939 to +59) was amplified using rat genomic DNA prepared from NRK52E cells and the following primers: sense, 5′-TCTTCTCCTAGTCTGTTG-3′ (−939 to −920) and antisense, 5′-AGAGATCTGCTCCAGTGAG-3′ (+40 to +59) and the following sense primers: for D1, 5′-CAGGGAATCTTGGAGCAAT-3′ (−759 to −730), for D2, 5′-CCAGGTT-GGAATTGACAA-3′ (−559 to −540), for D3, 5′-AGTATCTTCTCTCC-TTAGGA-3′ (−359 to −340), for D4, 5′-ATCTTCTGCTCAATGTCG-3′ (−219 to −200), for D5, 5′-ATCCGGCGGTCTCCTCTTCT-3′ (−179 to −160).

DNA transfection and luciferase assay

NRK52E cells were cotransfected with each firefly luciferase reporter construct and pRL (Renilla luciferase)-SV40 reporter vector (Promega) as a control of transfection efficacy. Cells were incubated in isotonic medium containing 5% FBS for 18 h, and were then treated with either fresh isotonic or hypertonic (200 mM mannitol) medium for 6 h (20). Cell lysates prepared with lysis buffer (Promega) were analyzed for firefly and Renilla luciferase activities using the Dual-Luciferase Reporter Assay System (Promega) in accordance with the manufacturer’s instructions, and the light emitted was measured by a MiniLumat LB 9506 luminometer (Berthold, Neustadt, NH). The firefly luciferase activity in relative light units was normalized against Renilla luciferase activity as a control, and osmotic response was calculated as the hypertonic-to-isotonic ratio. The average activity from three wells of six-well plates was used, and at least three independent experiments were performed. For statistical analysis, the mean ± SD was calculated.

EMSA

Nuclear extracts were prepared as described previously (20). After treatment with isotonic medium or NaCl (100 mM, 6 h), NRK52E cells were lysed in buffer composed of 10 mM HEPES/KOH (pH 7.9), 1.5 mM MgCl2, 0.1 mM KCl, 0.5 mM EDTA, 0.5% Nonidet P-40, and 0.5 mM PMSF, and were centrifuged at 14,000 × g for 5 min. The nuclear pellet was then resuspended in buffer containing 20 mM HEPES/KOH (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 25% glycerol; incubated for 45 min at 4°C; and centrifuged at 14,000 × g for 30 min at 4°C. The nuclear extract was stored at −80°C until use for EMSA.

A probe for the putative tonicity enhancer element (5′-TGGAAACAACCACCA-3′, MCP1-TonE/ORE) in the promoter region of the MCP1 gene was prepared by annealing two complementary oligonucleotides and end-labeled with [γ-32P]-ATP using T4 polynucleotide kinase. The probe (30,000 cpm) was incubated with 5 μg of the nuclear extract prepared above in reaction buffer containing 10 mM Tris/HCl (pH 7.5), 1.5 mM MgCl2, 0.5 mM EDTA, 0.5 mM DDT, 50 mM NaCl, 2 μg poly (dI-dC), and 4% glycerol. After incubation for 20 min at room temperature, the reaction products were subjected to 4% PAGE and visualized by autoradiography. For competition experiments, 100-fold excess of unlabeled oligonucleotides was added to the reaction mixture prior to the addition of labeled probe.

Measurement of MCP1 by ELISA

At the indicated time points after treatment of NRK52E cells with NaCl (100 mM) or mannitol (200 mM), aliquots of medium were centrifuged at 14,000 × g for 5 min at 4°C, and MCP1 levels in the supernatant were measured using an MCP1 Rat ELISA Biotrak system (GE Heathcare, Buckinghamshire, England). MCP1 levels were normalized against cell contents.
Results

Induction of MCP1 gene expression by NaCl and mannitol-induced hyperosmolality

In the first experiment, we used glucose (5–7), albumin (8), and LPS (9, 10) as positive controls that induce MCP1 mRNA expression. As shown in Fig. 1A, treatment of NRK52E cells with glucose (200 mM for 3 d), albumin (10 mg/ml for 24 h), or LPS (1 μg/ml for 24 h) resulted in significant elevation of MCP1 mRNA. Similarly, when NRK52E cells were treated with NaCl (100 mM) or mannitol (200 mM), MCP1 mRNA levels were markedly increased compared with treatment with isotonic medium. Treatment with NaCl or mannitol revealed that MCP1 was upregulated in a time-dependent manner by the NaCl–or mannitol-induced hyperosmotic stress response (Fig. 1B). NaCl (150 mM) or mannitol (300 mM) treatment at more than 600 mOsm/l resulted in decreased induction of MCP1 mRNA, compared with lower doses of NaCl and mannitol (Fig. 1C). It has also been reported that urea and glycerol elicit a hyperosmotic response in mouse renal medullary cells (23) and yeast Saccharomyces cerevisiae (25). However, in NRK52E cells, treatment with glycerol (200 mM) or urea (200 mM) failed to induce MCP1 gene expression (Fig. 1D).

Western blot analysis of MCP1 expression induced by NaCl and mannitol-induced hyperosmolality

To examine whether MCP1 protein synthesis is promoted by NaCl or mannitol-induced hyperosmolality, Western blot analysis was performed. In NRK52E cells treated with NaCl (100 mM; Fig. 2A), MCP1 protein was significantly increased, and higher levels remained at 24 h after treatment. Cells treated with mannitol (200 mM) also showed a marked elevation of MCP1 protein, which peaked at 12 h before decreasing (Fig. 2A). When NaCl or mannitol were applied to the cells at different doses, Western blot analysis showed no detectable expression of MCP1 protein at doses of more than 150 or 300 mM, respectively, suggesting that extreme hyperosmotic stress (>600 mOsm/l) suppresses MCP1 protein expression, possibly through translational inhibition (Fig. 2B). ELISA analysis of MCP1 in the culture medium revealed that MCP1 protein was released into the

FIGURE 1. RT-PCR analysis of MCP1 gene expression. A, Effect of glucose, albumin, LPS, NaCl, and mannitol on MCP1 expression in NRK52E cells. NRK52E cells were exposed to glucose (100 mM) for 3 d or albumin (10 mg/ml), LPS (1 μg/ml), NaCl (100 mM), or mannitol (200 mM) for 24 h. B, Time course of MCP1 expression under NaCl–or mannitol-induced hypertonic stress. NRK52E cells were treated with NaCl (100 mM) or mannitol (200 mM) for 24 h. Total RNA was prepared at the indicated time points and was subjected to RT-PCR. C, Effect of osmolality on NaCl– or mannitol-induced MCP1 expression. NRK52E cells were exposed to hypertonic medium at 400, 500, and 600 mOsm/l by addition of NaCl (50, 100, or 150 mM) or mannitol (100, 200, or 300 mM) for 3 h. D, Effect of glycerol and urea on MCP1 expression. NRK52E cells were exposed to NaCl (100 mM), glycerol (200 mM), or urea (200 mM) for 3 h. For each case, three independent experiments were performed. Iso, isotonic medium.
MCP1 mRNA expression.

Effects of protein synthesis inhibition

FIGURE 3. Western blot analysis of time course (A) and dose-dependency (B) of MCP1 protein expression induced by NaCl and mannitol hyperosmolality, and time course of MCP1 protein released into culture medium under hyperosmolality (C). A. NRK52E cells were treated with NaCl (100 mM) or mannitol (200 mM) for 6, 9, 12, 15, and 24 h, and cell lysates were subjected to Western blot analysis. In NRK52E cells treated with NaCl (100 mM), MCP1 protein was significantly elevated, and the levels remained higher at 24 h after treatment. Mannitol (200 mM) also induced a marked elevation in MCP1 protein reaching, which peaked at 12 h. B, NaCl (50, 100, 150, or 200 mM) or mannitol (100, 200, 300, or 400 mM) were applied to cells for 12 h. NaCl and mannitol induced marked MCP1 protein expression at doses of 100 and 200 mM, respectively. C, NRK52E cells were treated with NaCl (100 mM) or mannitol (200 mM) at the indicated time points, and MCP1 protein released into culture medium was measured by ELISA. **p < 0.01 versus Normal (6 or 24 h).

Signaling pathways participating in induction of MCP1 expression under hypertonic stress

It has been shown that inhibition of protein synthesis with CHX blocks hyperosmolality-induced gene expression in some cases (26), implicating de novo synthesized protein in gene expression regulation. In this study, MCP1 mRNA elevated by NaCl was suppressed by treatment with CHX (Fig. 3A). PKC and NF-κB signaling pathways have been demonstrated to be activated in cells cultured in medium containing high glucose (6, 7), high albumin (8), or high urea (27). However, calphostin C (a PKC inhibitor) and PDTC (an NF-κB inhibitor) showed no apparent inhibition of NaCl-induced MCP1 mRNA expression in NRK52E cells (Fig. 3B, 3C).

Several lines of evidence have demonstrated that MAPKs, such as p38MAPK (21, 22, 24), ERK (21–24) and JNK (21, 22, 24), are activated by hypertonic stress associated with NaCl. Thus, to further characterize the cell signaling pathways stimulated by hypertosmolality, involvement of MAPKs in the regulation of MCP1 expression was analyzed. Fig. 4A–C shows the time courses of activation of MAPKs after NaCl-hyperosmotic stimuli. NaCl-treated NRK52E cells exhibited time-dependent activation of MAPKs, namely p38MAPK, ERK, and JNK with their highest activation at 30 min after the treatment. This finding indicates that MAPK signaling pathways participate in the cellular response to hypertonic stress in NRK52E cells. To determine the inhibitory effects of each MAPK inhibitor on the activity of each MAPK, SB203580, PD98059, U0126, and SP600125 were used. Each inhibitor at the indicated doses markedly blocked MAPK activity (Fig. 4D–G).

In an additional experiment to clarify which MAPKs are involved in MCP1 regulation, the inhibitory effects of MAPK inhibitors on MCP1 mRNA expression were studied by RT-PCR analysis. Fig. 5 illustrates the effects of MAPK inhibitors on MCP1 gene expression. Treatment of NRK52E cells with SB203580 showed a marked decrease in MCP1 mRNA levels induced by hypertonic stress in a dose-dependent manner (Fig. 5A). PD98059 suppressed the increase in MCP1 mRNA associated with hypertonic stress (Fig. 5B). Similarly, U0126 also resulted in decreased MCP1 mRNA elevated by NaCl hypertonicity (Fig. 5B). Interestingly, the JNK inhibitor SP600125 failed to suppress the increase in expression of MCP1 by NaCl (Fig. 5C). In addition, curcumin, which abolishes AP1 activity (28), showed no suppressive effects on MCP1 mRNA expression (Fig. 5C), although curcumin at the same dose suppressed PMA-induced MCP1 expression, in which PMA stimulates the transcription of MCP1 through PKC and AP1 activation (29) (Fig. 5C). Hyperosmolar mannitol showed similar results to NaCl treatment (data not shown).

Furthermore, the effects of MAPK inhibitors on MCP1 protein expression were analyzed. As illustrated in Fig. 6, MAPK inhibitors, except for the JNK inhibitor, suppressed the increase in MCP1 protein levels by NaCl or mannitol in a dose-dependent manner, and
pertonic stress, the promoter region (hancer elements within the
A ss h o ni nF i g . 7
which contains the putative MCP1-TonE/ORE, was isolated, and
letion constructs, thus suggesting that there is a putative hypertonicity
a significant difference in luciferase activity between D4 and D5 de-
(9), AP1 (9) and Sp-1 (9)
Fig. 7
JNK-AP1 pathway, participate in cell signaling for
indicates that the p38MAPK and MEK–ERK pathways, but not the
activation of phosphorylated and nonphosphorylated
MCP1-ToE/ORE; 5
9
−TGGAAAAACACCAA-3
′, −199 to −186 bp
upstream of the transcription start site, there is a sequence (labeled
MCP1-ToE/ORE; 5′-TGGAAAAACACCAA-3′, −199 to −186)
similar to a uniform TonE/ORE consensus sequence (5′-(C/T)GG-
ANNN(C/T)N(C/T)-3′ (16) and/or 5′-NGGAAA(A/T)T(A/G)(C/A/
T)(A/C)-3′) (17, 18). Thus, to identify the cis-acting regulatory enhancer elements within the MCP1
MCP1-TonE/ORE, was isolated, and a series of deletion constructs were created for luciferase reporter assay. As shown in Fig. 7B, reporter assay with truncation constructs showed a significant difference in luciferase activity between D4 and D5 deletion constructs, thus suggesting that there is a putative hypertonicity
response element in the MCP1 promoter region similar to the TonE/
ORE identified previously.

Identification of TonE/ORE of MCP1
To elucidate the element responsive to hyperosmolality in the D4
construct, a synthesized oligonucleotide construct (D440) spanning
−219 to −180, including a putative toxicity response element (MCP1-
TonE/ORE), was inserted into the pGL3-SV40-promoter driven lucif-
erase reporter construct. As shown in Fig. 8A, the deletion constructs
of D440, D440M1, and D440M2 showed equal reporter activity to that
in the D440 construct. However, the D440M3 deletion construct with
an incomplete MCP1-TonE/ORE sequence was insensitive to hyper-
osmolality, suggesting that the putative toxicity response element
MCP1-TonE/ORE is a functional element responsive to hypertonicity.

The capacity of MCP1-TonE/ORE to behave as a toxicity-responsive element was further examined by subjecting the D440M2 construct to additional deletions and insertion into an SV40 promoter-driven lucif-
erase reporter construct. As shown in Fig. 8B, deletion of T at the 5′-end resulted in a slightly weaker reporter activity than the D440M2 construct (Fig. 8B; D440M2-D1). Further removal of 5′-TGG at the 5′-end completely abolished the osmotic response of the D440M2 construct (Fig. 8B; D440M2-D2). In addition, deletion of 5′-AAATTCCA at the 3′-end of D440M2, in which 5′-AA at the 3′-end of the MCP1-TonE/
ORE was removed, also diminished reporter activity to control levels.
HYPERTONICITY-INDUCED MCP1 EXPRESSION

Western blot and immunocytochemical analyses of nuclear translocation of TonEBP/OREBP/NFAT5

Western blot analysis showed that in NRK52E cells cultured in hypertonic medium with NaCl and/or mannitol, TonEBP/OREBP/NFAT5 was localized in nuclei in response to hypertonicity. Exposure to the MAPK inhibitors SB203580, PD98059, and U0126 resulted in a reduction in the nuclear TonEBP/OREBP/NFAT5 abundance caused by hypertonic stress (Fig. 10A).

Immunocytochemical observation also confirmed that the hypertonicity-induced nuclear translocation of TonEBP/OREBP/NFAT5 was suppressed by MAPK inhibitors, except for SP600125 (Fig. 10B).

Effect of dehydration on MCP1 expression and nuclear distribution of TonEBP/OREBP/NFAT5 in rat kidney in vivo

After treatment of rats with dehydration, immunoreactivity for MCP1 in normal kidney was not apparent, whereas MCP1 immunostaining in dehydrated rats was detected in tubular cells in the outer strip of the renal outer medulla (Fig. 11A). In addition, immunostaining of TonEBP/OREBP/NFAT5 revealed that nuclear abundance of TonEBP/OREBP/NFAT5 was increased in dehydrated rats, as compared with normal rats (Fig. 11B).

Discussion

In the inflammatory process, chemokines and cytokines play an important role in the pathophysiologic features of progressive diseases.
Among chemokines, MCP1 is a potent chemoattractant and activator for circulating monocytes/macrophages and T lymphocytes, and thus it has been implicated in the development of fibrotic diseases, such as liver fibrosis (31) and pulmonary fibrosis (32), as well as renal fibrosis and peritoneal fibrosis. For the prevention of organ dysfunction owing to fibrotic lesions, the molecular mechanisms underlying MCP1 regulation need to be understood at the molecular level. Previous studies have reported numerous stimuli that lead to expression of MCP1, including insulin (30), hypoxia/reoxygenation (33), mechanical stress (34), and advanced glycation end-product (35), as well as glucose, albumin and LPS. However, the regulation of MCP1 induction by hypertonicity alone remains poorly understood. In this study, we investigated the molecular regulation of MCP1 in response to hypertonicity.

In the first set of experiments, we examined the capacity of hypertonic NaCl or mannitol to induce MCP1 gene expression, in comparison with LPS, and glucose and albumin at high doses, as positive controls. Hyperosmolar NaCl and mannitol increased MCP1 mRNA levels in a time-dependent manner (Fig. 1). Furthermore, MCP1 protein synthesis and its release into the culture medium were elevated in a time-dependent manner by exposure to NaCl and mannitol (Fig. 2A, 2C). However, MCP1 protein production was significantly abolished by NaCl and mannitol at 600 mOsm/l (Fig. 2B). Previous studies have revealed that hypertonic NaCl at ~600 mOsm/l induces apoptosis (36) through mitochondrial dysfunction (37). Thus, the observed suppression of MCP1 protein synthesis might have resulted from such cellular damage when cells were exposed to the extremely high concentrations of NaCl and mannitol.

This study further investigated the intracellular signaling pathways participating in MCP1 expression in response to hypertonicity. In some genes, de novo protein synthesis has been found to regulate transcriptional activity (25, 38) and/or mRNA stability (39). In this study, MCP1 expression induced by NaCl was also inhibited by pretreatment with CHX, a translational inhibitor (Fig. 3A), thus suggesting that newly synthesized, hypertonicity-induced factors might be involved in the transcriptional activation of MCP1, although additional...

![FIGURE 8](http://www.jimmunol.org/)

**FIGURE 8.** Functional activity of MCP1-TonE/ORE under hypertonic stress. *A*, To confirm the location and functional activity of the osmotic responsive element in the promoter region of the MCP1 gene, a 40-bp fragment (D440) from -219 to -180 containing the TonE/ORE-like element 5’-TGGAAAAACACCAA-3’ (MCP1-TonE/ORE) was prepared based on the D4 construct, and its deletion mutant constructs (D440M1, D440M2, D440M3) were also inserted into the pGL3-SV40 promoter vector. There was a significant difference in luciferase activity between D440M2 and D440M3, suggesting that the D440M2 sequence is involved in the response to hyperosmolality. Underlining represents the consensus sequence to TonE/ORE. *B*, To characterize the functional activity of the D440M2 fragment, D440M2 was deleted. All deletion mutant constructs, except for D4M2-D3, failed to respond to hyperosmolality, suggesting that the D4M2-D3 sequence 5’-TGGAAAAACACCAA-3’ (MCP1-TonE/ORE) is a potential osmotic responsive element in the MCP1 gene. Underlining represents the consensus sequence to TonE/ORE. *C*, To further examine the activity of MCP1-TonE/ORE, constructs with multiple copies of MCP1-TonE/ORE were analyzed. Constructs having two (MCP1-TonE/ORE × 2) or three (MCP1-TonE/ORE × 3) copies of MCP1-TonE/ORE showed increased luciferase activity, as compared with the construct with a single copy of MCP1-TonE/ORE. Data in each panel represent mean ± SD, and the number of independent transfections for each construct was ≥3. *p* < 0.05; **p** < 0.01 versus SV40 promoter.

![FIGURE 9](http://www.jimmunol.org/)

**FIGURE 9.** EMSA of MCP1-TonE/ORE in MCP1 gene. A [32P]-labeled MCP1-TonE/ORE 14-bp oligonucleotide fragment was incubated with 5 μg of nuclear extracts prepared from NRK52E cells maintained in isotonic or hypertonic medium, in the absence (−) or presence of 100-fold excesses of unlabeled 14-bp oligonucleotide, as described in Materials and Methods. Probe, labeled 14-bp probe only; Isotonic, isotonic medium; Hypertonic, hypertonic medium supplemented with 100 mM NaCl; Competitor, unlabeled oligonucleotide fragment. The arrow indicates a slowly migrating band of MCP1-TonE/ORE and protein complex.
experiments are required to confirm whether this includes translation of TonEBP/OREBP/NFAT5.

Matsuo et al. (6) reported that hyperosmolar mannitol induced MCP1 mRNA and protein through the activation of PKC and NF-κB in peritoneal mesothelial cells. PKC activates NADPH-oxidase, leading to the production of reactive oxygen species, and in turn the reactive oxygen species activate NF-κB transcription factor through the phosphorylation of Ik-B, followed by proteasome degradation, which induces expression of NF-κB-regulated genes (8, 40). However, our results showed that the PKC inhibitor calphostin C, which broadly inhibits the activity of PKC subtypes, including α, β, γ, δ, and ε, does not suppress the increase in MCP1 mRNA caused by hyperosmotic NaCl (Fig. 3B). Moreover, the NF-κB inhibitor PDTC and the NADPH oxidase inhibitor DPI were also ineffective in attenuating the MCP1 expression induced by NaCl (Fig. 3C, D). Furthermore, the iron chelator DFO, which blocks the Fenton reaction leading to the production of H₂O₂ that activates NF-κB (40), also showed no suppressive effects on MCP1 mRNA levels (Fig. 3E). These results thus indicate that, PKC, NADPH oxidase, and H₂O₂, which are related to NF-κB activation, may not be involved in the signaling pathways underlying MCP1 induction in response to hyperosmolar NaCl or mannitol in NRK52E cells. This finding differs from the previous findings of Matsuo et al. (6) using peritoneal mesothelial cells, and it might be due to the difference in cell types used in this study, although further study is needed for clarification.

Recent studies on the adaptive mechanisms for hypertonicity have substantially improved our knowledge of hypertonicity-mediated intracellular signaling pathways and transcriptional regulation of tonicity-sensitive genes. In kidney tubular cells, such as Madin-Darby canine kidney cells (21), mouse inner medullary collecting duct cells (20, 22, 23), and rabbit kidney papillary epithelial (PAP-HT25) cells (24), p38MAPK, ERK, and JNK have been found to be involved in hypertonicity-mediated cell signaling cascades. The present study also demonstrated that hypertonic NaCl treatment results in the activation of p38MAPK, ERK, and JNK in NRK52E cells (Fig. 4), and MAPK inhibitors, except for the JNK inhibitor, abolished MCP1 mRNA (Fig. 5) and protein (Fig. 6) expression induced by NaCl.

**FIGURE 10.** Western blot (A) and immunocytochemical (B, C) analyses of nuclear translocation of TonEBP/OREBP/NFAT5. Nuclear distribution of TonEBP/OREBP/NFAT5 was suppressed by SB203580, PD98059 and U0126, but not SP600125. A, NRK52E cells were pretreated with SB203580 (5, 10, or 20 μM), PD98059 (20 μM), U0126 (5, 10, or 20 μM) or SP600125 (10 or 20 μM) for 1 h, followed by exposure to 100 mM NaCl and/or 200 mM mannitol for 6 h. Cell lysates were then subjected to Western blot analysis. B, NRK52E cells were pretreated with SB203580 (20 μM), PD98059 (20 μM), U0126 (20 μM), or SP600125 (20 μM) for 1 h, followed by hyperosmotic stress, were subjected to immunocytochemical observation using ImageXpress High Content Screening System (Molecular Devices, Sunnyvale, CA). N, normal; Na, NaCl (100 mM); Man, Mannitol (200 mM); SB, SB203580 (20 μM); PD, PD98059 (20 μM); U, U0126 (20 μM); SP, SP600125 (20 μM). **p < 0.01 versus Normal; #**p < 0.01 versus NaCl or Mannitol.

**FIGURE 11.** Effect of dehydration on MCP1 expression (A) and nuclear distribution of TonEBP/OREBP/NFAT5 (B) in rat kidney in vivo. a and c, Normal. b and d, Dehydration. Arrows indicate cytosolic MCP1 (a, b) and nuclear TonEBP/OREBP/NFAT5 staining (c, d). Dehydration resulted in elevated MCP1 protein expression and nuclear translocation of TonEBP/OREBP/NFAT5.
These results indicate that p38 MAPK and MEK–ERK, but not JNK, are involved in the toxicity-induced MCP1 expression in NRK52E cells. As shown in Fig. 6, although SB203580 inhibited equally MCP1 protein level induced by NaCl and mannitol, PD98059 and U0126 moderately suppressed the increased MCP1 protein by mannitol, as compared with the result with NaCl. A previous study has reported the endothelin synthesis by a differential activation of p38MAPK and ERK induced by hyperosmolar urea or betaine in Madin-Darby canine kidney cells (41), suggesting a differential participation of urea and betain via p38MAPK and ERK activation in the cellular event. Our result also suggests that in NRK52E cells NaCl and mannitol might differentially participate in MCP1 expression through p38MAPK and MEK–ERK signaling pathways with a different degree, although transcriptional and translational analyses are further needed to demonstrate the molecular mechanisms of the regulation of MCP1 synthesis.

The toxicity-responsive element Ton/EORE has been identified in the 5′-flanking region of toxicity-sensitive genes (11, 12). Luciferase reporter assay using the MCP1 promoter region showed significant activity in luciferase reporter constructs containing the Ton/EORE consensus sequence from the MCP1 gene (Fig. 7B). Moreover, based on experiments with deletion mutant constructs (Fig. 8), 5′-TGGAAAACACCAA-3′ is an essential sequence for functional activity in response to hypertonicity and falls within the uniform consensus sequence of Ton/EORE, 5′-(C/T)GGAANNN(C/T)N(C/T)-3′ (16), or 5′-NGGAAA(A/T)(C/A/G)(C/A/T)(A/C)-3′ (17, 18). When compared with the transcriptional activity between MCP1-Ton/EORE ×2 and ×3, the activities were almost equivalent (Fig. 8). This finding might show that two copies of the elements are sufficient to drive a maximum transcriptional activity or that the observed equivalent activity is due to the impaired efficiency of protein synthesis by hypertonic stress, although the molecular mechanism by which hypertonicity inhibits translation is not well understood (42). In addition, the API/Sp-1 element of the MCP1 gene was insensitive to hyperosmolality because the D5 deletion construct containing the API/Sp-1 element failed to increase reporter activity (Fig. 7B). This observation was consistent with the result shown in Fig. 5C, in which inhibition of JNK failed to block the elevation of MCP1 mRNA by NaCl-hypertonicity, indicating that the JNK-API signaling pathway is not involved in hypertonicity-mediated MCP1 regulation in NRK52E cells. Our EMSA experiment showed a slowly migrating band for the MCP1-Ton/EORE probe in cells exposed to hypertonic NaCl (Fig. 9), indicating that MCP1-Ton/EORE is a functional enhancer in response to hypertonicity.

TonEBP/OREBP/NFAT5, a rel/NF-kB family member, has been identified as a transcription factor that activates transcription of osmosensing genes in response to hypertonicity (13–15). TonEBP/OREBP/NFAT5 has been also found to be widely distributed in various tissues, including the kidney (13), and is translocated into the cellular nucleus (13, 14) by a nuclear localization signal (43) under hypertonic stimulation. As shown in Fig. 10, p38MAPK and MEK–ERK inhibitors, but not the JNK inhibitor, blocked the nuclear translocation of TonEBP/OREBP/NFAT5 induced by NaCl or mannitol, suggesting that p38MAPK and MEK–ERK signaling pathways (44, 45) are associated with the nuclear distribution of TonEBP/OREBP/NFAT5 mediated by hypertonicity. This result is also consistent with the effects of MAPK inhibitors on MCP1 mRNA and protein levels, as shown in Figs. 5 and 6, which suggests that MCP1 gene expression caused by hypertonicity with NaCl and mannitol is driven by TonEBP/OREBP/NFAT5.

It has been reported that dehydration upregulates the expression of toxicity-sensitive genes, such as an ion channel (46), a solute transporter (47), a metabolic enzyme (48), and heat shock proteins (26, 49) in the kidney in vivo. In vivo dehydration also induces the nuclear distribution of TonEBP/OREBP/NFAT5 in thick ascending limbs and the S3 segment (pars recta) of proximal tubules in the rat kidney (50). In the current study, dehydrated rats showed increased MCP1 protein expression in the kidney, which is known to experience an increase in tissue osmolality under such conditions. There was also an increase in nuclear translocation of TonEBP/OREBP/NFAT5 in rat kidneys during dehydration (Fig. 11), suggesting that MCP1 is induced by hypertonicity in the in vivo kidney, as well as in cultured cells, through the nuclear distribution of TonEBP/OREBP/NFAT5.

Previous studies reported that hypertonicity induces expression of cytokines, such as IL-8 (51) and TNF-α (52, 53), in PBMCs, T cells, and fibroblasts; however, the biologic significance of their accelerated expression is poorly understood. Sugiuara et al. (54) showed that hypertonic stress activated the processing of latent TGF-β to the biologically active form in cultured fibroblast cells, leading to the stimulation of collagen synthesis that mediates fibrotic lesions. The results of this study suggest that hypertonicity can activate proinflammatory cascades in the kidney by promoting cytokine production. In contrast, TNF-α has been found to stimulate the transcriptional activation of an osmoprotective gene, the AR gene, through the Ton/EORE enhancer element (55), suggesting that cytokine induction by hypertonicity can contribute to osmoprotective adaptation. Further study is needed to investigate the biologic significance of MCP1 expression in response to hypertonicity in regard to osmoprotection or pathogenesis associated with tissue fibrosis.

The results of this study indicate that in NRK52E cells, NaCl, and mannitol significantly induce MCP1 mRNA and protein production, and the p38MAPK and MEK–ERK signaling pathways are involved in the regulation of MCP1 gene expression. This study further identified a hypertonicity-sensitive cis-acting element, MCP1-Ton/EORE, in the 5′-flanking region of the MCP1 gene, and MCP1-Ton/EORE is transactivated through the TonEBP/OREBP/NFAT5, which is activated by the p38MAPK and MEK–ERK signaling pathways under hypertonic stress. In addition, in vivo dehydration induces expression of MCP1 in the rat kidney.

Disclosures
The authors have no financial conflicts of interest.

References


