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High glucose accelerates MCP-1 production via p38 MAPK in vascular endothelial cells

Hiroshi Takaishi,[a] Takahiro Taniguchi,[a,*] Akihiro Takahashi,[a] Yuichi Ishikawa,[b] and Mitsuhiro Yokoyama[a]

[a] *Division of Cardiovascular and Respiratory Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan*
[b] *Faculty of Health Sciences, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan*

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Abstract

In diabetes mellitus (DM), hyperglycemia causes cardiovascular lesions through endothelial dysfunction. Monocyte chemoattractant protein-1 (MCP-1) is implicated in the pathogenesis of cardiovascular lesions. By using human umbilical vein endothelial cells, we investigated the effect of hyperglycemia on MCP-1 production and its signaling pathways. Chronic incubation with high glucose increased mRNA expression and production rate of MCP-1 in a time (1–7 days)- and concentration (10–35 mM)-dependent manner. Chronic exposure to high glucose resulted in enhancement of generation of reactive oxygen species (ROS), as determined by increasing level of 2,7-dichlorofluorescein (DCF), and subsequent activation of p38 mitogen-activated protein kinase (MAPK). Neither c-Jun NH_2-terminal kinase nor extracellular signal-regulated kinase1/2 was affected. SB203580 or FR167653, p38 MAPK specific inhibitors, completely suppressed MCP-1 expression. Catalase suppressed p38 MAPK phosphorylation and MCP-1 expression. These results indicate that hyperglycemia can accelerate MCP-1 production through the mechanism involving p38 MAPK, ROS-sensitive signaling pathway, in vascular endothelial cells.

Keywords: Monocyte chemoattractant protein-1; Human umbilical vein endothelial cells; p38 Mitogen-activated protein kinase; Reactive oxygen species

Cardiovascular complications are the leading cause of morbidity and mortality in patients with diabetes mellitus (DM) [1,2]. Because the onset and progression of complications are delayed in patients with good glycemic control [3], hyperglycemia is thought to be an important regulator of vascular lesion development. Elevated glucose concentrations can induce dysfunction of several intracellular signal transduction cascades, including modulation of protein kinase C (PKC) [4,5], generation of reactive oxygen species (ROS) [6–8], and accumulation of advanced glycation end products (AGEs) [9]. A recent study demonstrated that hyperglycemia-induced mitochondrial superoxide overproduction might be the unifying pathway that activates PKC activity, increases intracellular sorbitol and AGE formation, and subsequently induces endothelial cell dysfunction [8]. However, the underlying mechanisms between hyperglycemia and vascular disease development were not fully clarified.

Monocyte chemoattractant protein-1 (MCP-1), a C–C chemokine, controls chemotaxis of mononuclear cells [10,11]. MCP-1 and its receptor (CCR2) pathway recently attracted much attention, because the MCP-1 seems to be involved in the inflammatory aspect of atherogenesis. Atheroma-forming cells (endothelial cells, smooth muscle cells, and macrophages) express MCP-1 and activity of MCP-1/CCR2 pathway is increased in atherosclerotic lesions [12–14]. Furthermore, MCP-1 induces adhesion molecules [15], proinflammatory cytokines [15,16], chemokines, MMP [17], and tissue factor expression [18]. These findings suggest that MCP-1 contributes to the initiation and development of cardiovascular lesions.

* Corresponding author. Fax: +81-78-382-5859.
E-mail address: taniguch@med.kobe-u.ac.jp (T. Taniguchi).

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The members of mitogen-activated protein kinases (MAPKs) play a crucial role in the regulation of MCP-1 production. Numerous studies have implicated ROS as participants in a variety of intracellular signaling sequences including members of MAPK [19–21] and other signaling systems [22].

Because the prevalence of cardiovascular complications is significantly greater in diabetic patients than in nondiabetic subjects, we hypothesized that MCP-1 may be preferentially activated in the setting of diabetes and that hyperglycemic environment could regulate the MCP-1 production from the vascular cells.

In the present study, we documented the fact that human vascular endothelial cells, chronically exposed to high glucose, generated intracellular hydrogen peroxide (H_2O_2) and highly expressed MCP-1 through the mechanism involving p38 MAPK pathway, a member of the MAPK family and a component of oxidant stress-sensitive signaling pathway.

Materials and methods

Cell culture and experimental design. Human umbilical vein endothelial cells (HUVECs) were isolated from at least three healthy volunteers and used between passages 2 and 4. HUVECs were cultured on 0.25% gelatin coated dishes at 37 °C, 5% CO_2 in RPMI1620 medium (Sigma chemicals, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 20 μg/ml endothelial cell growth supplement (ECGS) (Becton–Dickinson), 5 U/ml heparin, 50 U/ml penicillin, and 50 μg/ml streptomycin (Sigma). After HUVECs were grown under confluency, the cells were maintained in the culture medium that contained either 10 mM D-glucose (as a control), 25 and 35 mM D-glucose (as a high glucose), or 25 mM D-mannitol (as an osmotic control for high glucose) for up to 7 days. The conditioned media were refreshed everyday. To investigate the effects of p38 MAPK inhibitor, SB203580 (Calbiochem, CA) or FR167653 (Fujisawa Pharmaceutical), PKC inhibitor, Bisindolylmaleimide I(GF109203X) (Calbiochem), and MEK1/2 inhibitor (U10260) (Calbiochem), the cells were treated with these reagents for 6 h prior to harvesting. To investigate the effects of antioxidants, vitamin C (Sigma), *N*-acetylcysteine (Sigma), and catalase (Roche) were administrated for the last 24 h.

Detection of reactive oxygen species production. To detect intracellular production of reactive oxygen species (ROS), we employed carboxy-2′,7′-dichlorodihydrofluorescein diacetate (Carboxy-H_2DCFDA, Molecular Probes). The dye is cleaved and trapped intracellularly, where it can be oxidatively modified by intracellular H_2O_2 and peroxidases to produce fluorescent 2′,7′-dichlorofluorescein (DCF). HUVECs were treated with different glucose concentrations for predetermined periods, or H_2O_2 (500 μM) for 15 min, and then exposed to 20 μM Carboxy-H_2DCFDA in phosphate buffered saline (PBS) for 15 min. The cells were harvested by trypsinization and then collected by centrifugation, washed twice with PBS, and resuspended in PBS containing 2 μg/ml propidium iodide. Viable cells were sorted out and the intensity of DCF in the cells was analyzed by FACScan flow cytometry (Becton–Dickinson) and Cell Quest software (Becton–Dickinson).

RNA preparation and Northern blot analysis of MCP-1 mRNA. Total RNA was extracted using ISOGEN reagent (Nippon Gene, Japan), according to the manufacturer's protocol. The amount of mRNA was quantified by Northern blot analysis. Total RNA (10 μg/lane) was electrophoresed on 1% formaldehyde–agarose gels in the presence of ethidium bromide. The RNA was transferred to a nitrocellulose membrane (Hybond N, Amersham Pharmacia Biotech) by capillary action and covalently linked by UV irradiation. Human MCP-1 cDNA was labeled with [α-^{32}P]dCTP using random primer labeling kits (Amersham Pharmacia Biotech). Hybridization was performed with labeled human MCP-1 cDNA probe for 2 h at 68 °C. After hybridization, membranes were washed twice with 1× SSC and 0.1% SDS and then washed once with 0.1× SSC and 0.1% SDS at 68 °C for 20 min. The level of MCP-1 mRNA was quantified from the radioactivity by using bio-image analyzer (Fujix BAS 2000 system).

Measurement of MCP-1 protein in the medium. The cultured medium of HUVECs was exchanged every 24 h for 7 days and collected at each time. The concentration of the MCP-1 protein in the collected medium was quantified by using a commercial solid phase enzyme linked immunosorbent assay (ELISA) (Biosourse International, Camarillo, CA).

Western blot analysis. HUVECs were rinsed twice with chilled PBS and lysed by scraping in a lysis buffer containing 20 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 0.1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 0.15 U/ml aprotinin. The proteins (20 μg/well) were separated by using 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gels under reducing conditions and transferred onto microporous polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Tokyo). Membranes were blocked with 5% skim milk in TBS-T (TBS with 0.1% Tween 20, pH 7.6) for 1 h and thereafter immunoblotted with anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-ERK1/2, anti-ERK1/2, and anti-phospho-JNK polyclonal antibodies (New England Biolabs, Beverly, MA), respectively. A horseradish peroxidase-linked anti-rabbit IgG antiserum was employed as a secondary antibody and the bands of interest were detected using an enhanced chemiluminescence (ECL) technique (Amersham Pharmacia Biotech).

Assay of p38 MAPK kinase activity. HUVECs were rinsed twice with chilled PBS and incubated in 0.5 ml ice-cold cell lysis buffer plus 1 mM PMSF for 5 min. The lysis buffer contained 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1 mM Na_3VO_4, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, and 1 μg/ml leupeptin. The tissues were sonicated four times for 5 s each on ice and centrifuged for 10 min at 4 °C. The supernatant was stored at −70 °C for activity assay.

For immunoprecipitation of phosphorylated p38 MAPK, 200 μl cell lysate containing 200 μg total protein was incubated with 20 μl of resuspended immobilized phospho-p38 MAPK (Thr180/Tyr182) monoclonal antibody (New England Biolabs) overnight at 4 °C. The samples were then centrifuged for 30 s at 4 °C and the pellet was washed twice with 500 μl lysis buffer and washed twice with 500 μl kinase buffer containing 20 mM Tris (pH 7.5), 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$. Immune complexes were resuspended in 50 μl kinase buffer supplemented with 200 μM ATP and 2 μg of activating transcription factor-2 (ATF-2, New England Biolabs) for 30 min at 30 °C. The reaction was terminated with 25 μl of 3× SDS sample buffer containing 187.5 mM Tris–HCl (pH 6.8 at 25 °C), 6% (w/v) SDS, 30% glycerol, 150 mM DTT, and 0.03% (w/v) bromophenol blue. The samples were boiled for 5 min and loaded on 10% SDS–PAGE gels. Phosphorylation of ATF-2 protein at Thr71 was measured by Western blot analysis using a phospho-ATF-2 (Thr71) polyclonal antibody.

Statistical analysis. Results are expressed as means ± SEM. The significance of differences among experimental groups was determined by ANOVA. Statistical significance was assumed at $p < 0.05$ and $p < 0.01$.

Results

Chronic exposure to high glucose enhanced the expression of MCP-1 mRNA and production rate of MCP-1 protein in HUVECs

Although exposure of HUVECs to high glucose for 24 h did not enhance the expression of MCP-1 mRNA, chronic exposure to high glucose for up to 7 days significantly led to the enhancement of MCP-1 mRNA expression in a concentration (10–35 mM glucose)-dependent manner (Fig. 1A). To exclude the osmolal effect of glucose from the expression of MCP-1 mRNA, HUVECs were cultured with high concentration of D-mannitol (25 mM) for 7 days. As shown in Fig. 1A, high mannitol had no effect on the expression of MCP-1 mRNA. In accordance with these results, exposure to high glucose but not high mannitol increased the amount of MCP-1 protein secreted into the cultured medium in a concentration- and a time (1–7 days)-dependent manner (Fig. 1B).

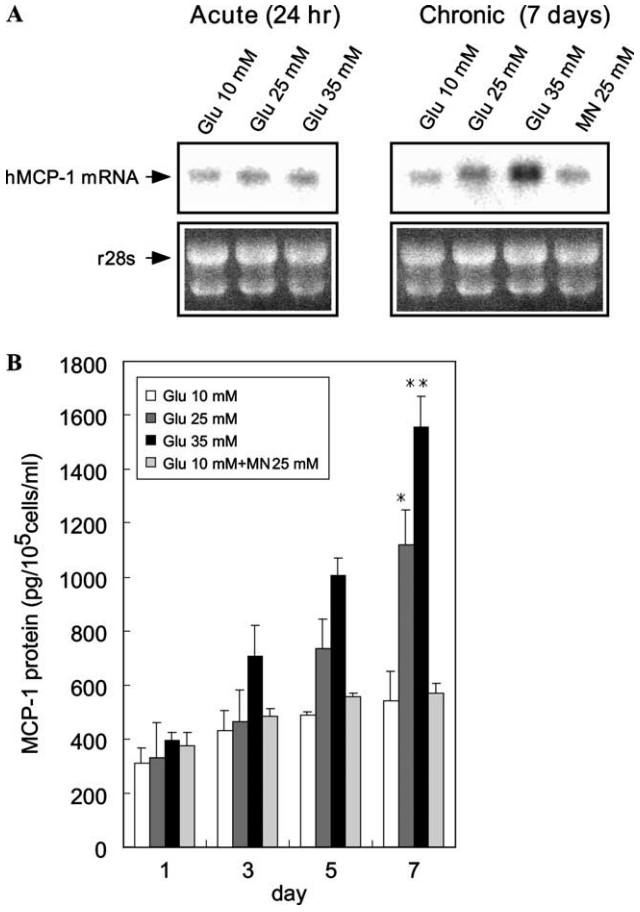

Fig. 1. Increase of expression of MCP-1 mRNA and production rate of MCP-1 protein in HUVECs exposed to high glucose. (A) HUVECs were cultured for up to 7 days under normal glucose (10 mM), high glucose or mannitol (10 mM glucose + mannitol 25 mM) (MN) medium. The steady-state expression of MCP-1 mRNA at indicated periods was quantified by Northern blot analysis. The result shown represents one of the three independent experiments. (B) HUVECs were cultured for up to 7 days in indicated concentrations of glucose or mannitol and MCP-1 protein levels in the medium, exchanged, and harvested every 24 h, were determined by ELISA. $N = 3$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. normal glucose (10 mM).

Chronic exposure to high glucose influenced the phosphorylation status of MAP kinase members in HUVECs

To determine the potential involvement of MAPK family pathway in MCP-1 production in HUVECs exposed to high glucose, we surveyed the phosphorylation status of the MAPK family. Acute exposure (for 24 h) to high glucose (35 mM) slightly increased ERK1/2 (Thr202/Tyr204) phosphorylation, though longer exposure (for 7 days) to high glucose (10 and 35 mM) did not enhance the phosphorylation (Fig. 2A). JNK (Thr183/Tyr185) phosphorylation was not observed. In contrast, p38 MAPK were significantly phosphorylated by longer exposure to high glucose (Fig. 2A). Additionally, p38 MAPK (Thr180/Tyr182) phosphorylation increased in a concentration (10–35 mM)-dependent manner. However, high mannitol (25 mM) had no effect on phosphorylation of p38 MAPK in HUVECs.

HUVECs were incubated with high glucose (35 mM) for up to 7 days. p38 MAPK phosphorylation increased in a time-dependent manner (Fig. 2B). To confirm p38 MAPK activation, the p38 MAPK were immunoprecipitated from HUVEC lysates exposed to high glucose and in vitro kinase assays were performed by using ATF-2 as a substrate. As revealed using a specific anti-phospho ATF-2 antibody, p38 MAPK activity also increased in accordance with increasing phosphorylation of p38 MAPK (Thr180/Tyr182) (Fig. 2B).

p38 MAPK pathway was involved in MCP-1 mRNA expression induced by high glucose in HUVECs

To determine the involvement of p38 MAPK pathway on MCP-1 expression induced by chronic exposure to high glucose, we examined the effect of pharmacological kinase inhibitors, namely selective p38 MAPK inhibitors, SB203580 and FR167653 [23–25], a MEK1/2 inhibitor U0126, and a general PKC inhibitor GF109203X. Both SB203580 and FR167653 significantly inhibited the expression of MCP-1 mRNA and the effects were concentration-dependent (Fig. 3B). U0126 10 μM, presumed to fully block the MEK1/2 activation, had no effect on MCP-1 expression, besides paradoxically increasing the MCP-1 expression (Fig. 3A). Because high glucose environment increases PKC activity in vitro and in vivo [4,5] and p38 MAPK activation is reported to be involved in PKC pathway in other cell [26], we tested the effect of PKC inhibitor on MCP-1 mRNA expression induced by chronic exposure to high glucose. GF102903X 10 μM, estimated enough

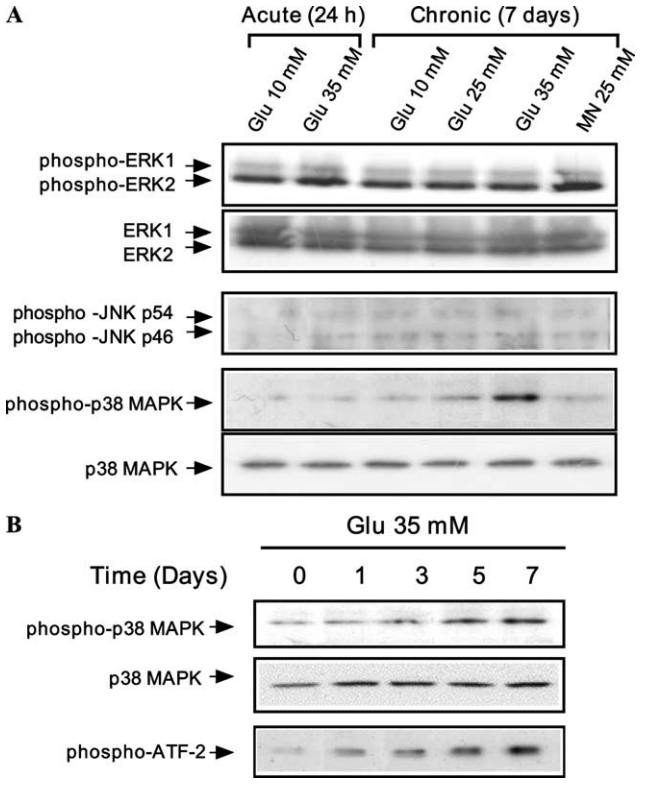

Fig. 2. p38 MAPK phosphorylation and activation in HUVECs exposed to high glucose. (A) HUVECs were cultured for up to 7 days under normal glucose (10 mM), high glucose or mannitol (10 mM glucose + mannitol 25 mM) (MN) medium. The phosphorylation status of MAPK family at indicated periods was quantified by immunoblot analysis. Cell lysates were analyzed by immunoblotting with the phospho-specific anti-bodies. (B) HUVECs were cultured for various periods of time as indicated in high glucose (35 mM) medium. The phosphorylation status of p38 MAPK was quantified in cell lysates of the HUVECs. ATF-2 was reacted with immunoprecipitated p38 MAPK from the HUVEC lysates in vitro. The phosphorylation status of ATF-2 was representative of p38 MAPK activity. The result shown represents one of the three independent experiments.

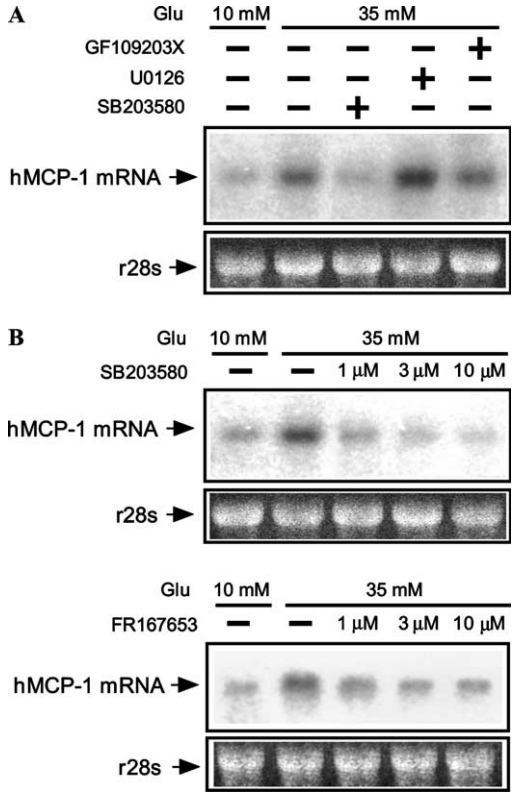

Fig. 3. Involvement of p38 MAPK pathway in the expression of MCP-1 mRNA in HUVECs exposed to high glucose. (A) HUVECs cultured for 7 days in high glucose (35 mM) medium were treated with SB203580 (10 μM), U0126 (10 μM) or GF102903X (10 μM) for the last 6 of the incubation. (B) Various concentrations of SB203580 or FR167653 as indicated were administer for the last 6 h of the incubation. Expression of MCP-1 mRNA was quantified by Northern blot analysis. The result shown represents one of the three independent experiments.

to block activities of novel and conventional PKC isoforms, had no effect on p38 MAPK phosphorylation (Thr180/Tyr182) (data not shown) and MCP-1 mRNA expression (Fig. 3A).

Chronic exposure to high glucose induced reactive oxygen species in HUVECs

To investigate whether ROS was generated in HUVECs exposed to high glucose, cells were analyzed by intracellular staining for ROS using H_2DCFDA, a specific fluorescent dye for hydrogen peroxide (H_2O_2). The increased level of fluorescein was measured by flow cytometry. Chronic but not acute (24 h) exposure to high glucose induced generation of intracellular H_2O_2 in a time (1–7 day)- and a concentration (10–35 mM)-dependent manner (Fig. 4), while high mannitol (25 mM) had no effect on the generation of intracellular H_2O_2 (data not shown).

A H_2O_2 scavenger suppressed p38 MAPK phosphorylation and expression of MCP-1 mRNA induced by high glucose

To assess whether the generation of intracellular ROS induced by high glucose involves the activation of p38 MAPK pathway, we examined the effect of antioxidants. HUVECs were treated with *N*-acetylcysteine (20 mM), vitamin C (100 μM), and catalase (6000 U/ml) for the last 24 h of 7 days high glucose environment, respectively. Catalase, a H_2O_2 scavenger, inhibited p38 MAPK phosphorylation, whereas neither *N*-acetylcysteine nor vitamin C appeared to inhibit the p38 MAPK phosphorylation (Fig. 5A). These results suggest that intracellular H_2O_2 might be a mediator for the stimulation of p38 MAPK signal transduction pathway. As expected from the inhibitory effect of p38 MAPK, catalase also suppressed MCP-1 mRNA expression (Fig. 5B).

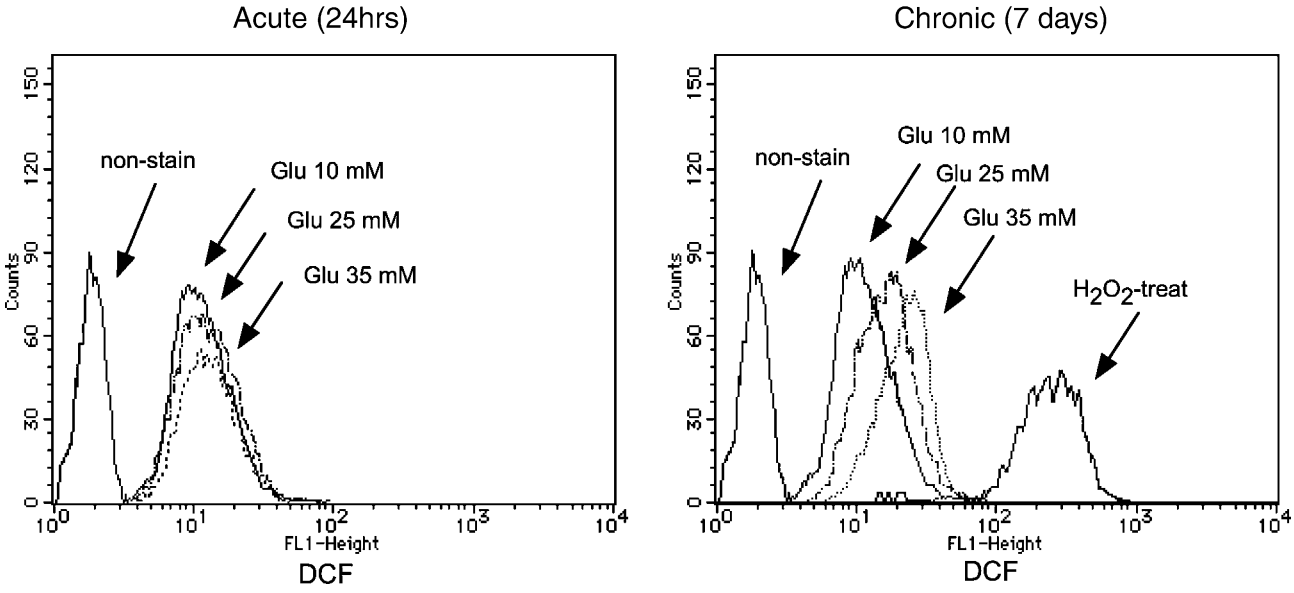

Fig. 4. Production of reactive oxygen species in HUVECs exposed to high glucose HUVECs (35 mM) were cultured for up to 7 days under normal glucose (10 mM) or high glucose medium. HUVECs at indicated periods were stained with Carboxy-H_2DCFDA (20 μM) for 15 min. Amount of ROS was determined by FACS analysis. H_2O_2 (500 μM for 15 min before staining) was used as a positive control. The histogram shown represents one of the three independent experiments.

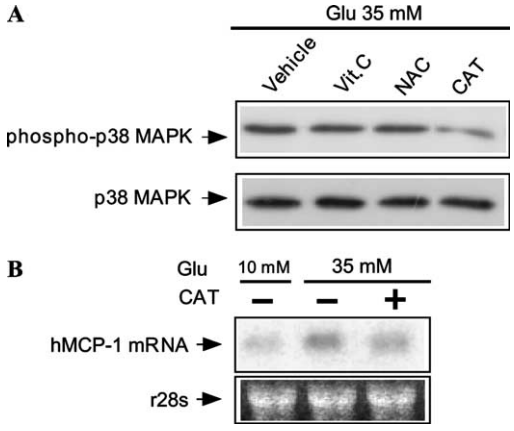

Fig. 5. Role of reactive oxygen species in high glucose-mediated p38 MAPK activation. (A) HUVECs cultured for 7 days in high glucose (35 mM) medium were treated with 20 mM *N*-acetylcysteine (NAC), 100 μM vitamin C (Vit.C) or 6000 U/ml catalase (CAT) for the last 24 h of the incubation. The phosphorylation status of p38 MAPK was quantified in cell lysates of the HUVECs. (B) Catalase (6000 U/ml) was administrated for the last 24 h of the incubation. Expression of MCP-1 mRNA was quantified by Northern blot analysis. The result shown represents one of the three independent experiments.

Discussion

It has been reported that high glucose generates mitochondrial superoxide anion in vascular endothelial cells [6,8]. In addition, increased flux of glucose through the polyol pathway reduces antioxidant enzyme activity depleting NADPH in vascular cells. Thus high glucose environment can enhance the generation of ROS in vascular cells [7]. There seems to be an association between ROS generation induced by high glucose and development of vascular complications but the underlying mechanisms have not been fully documented. In the present study, we demonstrated that chronic exposure to high glucose induced accumulation of intracellular hydrogen peroxide in HUVECs and accelerated MCP-1 production by activation of p38 MAPK, a component of oxidant stress-sensitive signaling pathway.

MCP-1, a member of the C–C chemokine family, attracts blood monocytes both in vitro and in vivo [27,28] and triggers their adhesiveness and transmigration through the endothelial layer. MCP-1 has a key role in the initiation and development of cardiovascular lesions [13]. Accumulating evidences revealed the fact that in human endothelial cells, MKK/p38 MAPK pathway rather than Raf/MEK/ERK or SEK/JNK pathway plays a crucial role in inflammatory cytokine-induced MCP-1 production [23,24,29,30]. p38 MAPK is highly activated on exposure to inflammatory cytokines such as TNF-α and IL-1 and a wide variety of environmental stress factors such as lipopolysaccharides (LPS), arsenite, anisomycin, and ultraviolet (UV) light, and also oxidative stress [21,26,31,32].

Our results indicated that chronic incubation with high glucose accumulated ROS in a time (1–7 days)- and concentration (10–35 mM)-dependent manner, as measured by the fluorescent activity of DCF in HUVECs. The oxidant species responsible for acceleration of MCP-1 production is likely to be H_2O_2. This is supported by the following observations: (I) fluorescent activity of DCF, H_2O_2-sensitive probe [33,34], increased

in a time (1–7 days)- and concentration (10–35 mM)-dependent fashion (Fig. 4); (II) p38 MAPK and ERK1/2 signaling pathways are ROS-sensitive. ERK1/2 is highly sensitive to superoxide [35] and p38 MAPK has increased sensitivity to H_2O_2 in vascular smooth muscle cells [21,31]. In the present study, p38 MAPK but not ERK1/2 was phosphorylated and activated in a time- and concentration-dependent fashion (Fig. 3B); and (III) catalase, which can inactivate H_2O_2 [30], suppressed phosphorylation of p38 MAPK and expression of MCP-1 mRNA (Fig. 5).

Hyperglycemia activates various PKC isoforms [4,5]. To investigate whether PKC pathway is involved in MCP-1 mRNA expression and p38 MAPK activation in HUVECs, we administer the general PKC antagonist GF109203X. As indicated, the administration of PKC antagonist had no effect on MCP-1 mRNA expression (Fig. 3A) and p38 MAPK phosphorylation (data not shown). Some PKC species have been demonstrated to activate specific NAD(P)H oxidase subunits and to regulate subsequent superoxide anion production in vascular cells [36]. However in the present study, the administration of the PKC antagonist did not affect the generation of intracellular H_2O_2 (data not shown). As discussed above, the oxidant species responsible for MCP-1 mRNA expression through p38 MAPK activation may offer some explanation.

The generation of ROS associated with hyperglycemia may also be due to increased concentrations of AGEs. AGEs have been demonstrated to interact with specific receptors and induce oxidative stress, enhance vascular cell adhesion molecule-1 expression and increase endothelial adhesiveness of monocytes [37]. The relatively short-time course used in our experiments has been demonstrated to result in a slight elevation of AGEs [38]. In the present study, their role might not be fully excluded.

In this study, we presented the inhibitory effect of catalase, but not *N*-acetylcysteine and vitamin C, on phosphorylation of p38 MAPK in high glucose-stimulated HUVECs (Fig. 5). Recent studies showed that *N*-acetylcysteine had paradoxical effect to enhance LPS activity in serum-containing media [39], and high concentration of vitamin C directly activated p38 MAPK in HUVECs [40]. Our results also did not clarify the inhibitory effect of *N*-acetylcysteine and vitamin C as an antioxidant on p38 MAPK phosphorylation.

In conclusion, the results in the present study demonstrated that high glucose induced MCP-1 production in human vascular endothelial cells and provided the possible signaling pathway of high glucose-induced MCP-1 expression, in which ROS generation and p38 MAPK activation are involved in MCP-1 expression. These findings indicate that in diabetes mellitus, sustained hyperglycemia can accelerate MCP-1 production from vascular endothelial cells through the mechanism involving ROS and p38 MAPK, and might accelerate the development of vascular lesions.

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