Journal of Cellular Biochemistry 88:1235–1246 (2003)

Involvement of MEKK1/ERK/P21$^{Waf1/Cip1}$ Signal Transduction Pathway in Inhibition of IGF-I-Mediated Cell Growth Response by Methylglyoxal

Jun Du,[1]* Shaohui Cai,[2,3] Haruhiko Suzuki,[2] Anwarul A. Akhand,[2] Xiuyang Ma,[1] Yoshikazu Takagi,[1] Toshio Miyata,[4] Izumi Nakashima,[2] and Fumihiko Nagase[1]

[1]Department of Medical Technology, Nagoya University School of Health Sciences, Aichi 461-8673, Japan
[2]Department of Immunology, Nagoya University Graduate and Faculty School of Medicine, Aichi 466-8550, Japan
[3]College of Pharmacy, Jinan University, Guangzhou 510612, China
[4]Institute of Medical Sciences and Department of Internal Medicine, Tokai University of Medicine, Kanagawa 259-1193, Japan

Abstract The abnormal accumulation of methylglyoxal (MG), a physiological glucose metabolite, is strongly related to the development of diabetic complications by affecting the metabolism and functions of organs and tissues. These disturbances could modify the cell response to hormones and growth factors, including insulin-like growth factor-1 (IGF-I). In this study, we investigated the effect of MG on IGF-I-induced cell proliferation and the mechanism of the effect in two cell lines, a human embryonic kidney cell line (HEK293), and a mouse fibroblast cell line (NIH3T3). MG rendered these cells resistant to the mitogenic action of IGF-I, and this was associated with stronger and prolonged activation of ERK and over-expression of P21$^{Waf1/Cip1}$. The synergistic effect of MG with IGF-I in activation of ERK was completely abolished by PD98059 but not by a specific PI3K inhibitor, LY294002, or a specific PKC inhibitor, bisindolylmaleimide. Blocking of Raf-1 activity by expression of a dominant negative form of Raf-1 did not reduce the enhancing effect of MG on IGF-I-induced activation of ERK. However, transfection of a catalytically inactive form of MEKK1 resulted in inactivation of the MG-induced activation of ERK and partial inhibition of the enhanced activation of ERK and over-expression of p21$^{Waf1/Cip1}$ induced by co-stimulation of MG and IGF-I. These results suggested that the alteration of intracellular milieu induced by MG through a MEKK1-mediated and PI3K/PKC/Raf-1-independent pathway resulted in the modification of cell response to IGF-I for p21$^{Waf1/Cip1}$-mediated growth arrest, which may be one of the crucial mechanisms for MG to promote the development of chronic clinical complications in diabetes. J. Cell. Biochem. 88: 1235–1246, 2003.

Key words: IGF-I; methylglyoxal; MEKK1; ERK; p21$^{Waf1/Cip1}$

A variety of glucose metabolites have been found to be abnormally accumulated in diabetes subjects due to a disorder in glucose metabolism, and this abnormal accumulation of glucose metabolites might accelerate the onset and progression of diabetic complications [Trial Research Group, 1993; Prospective Diabetes Study (UKPDS) Group, 1998; Brownlee, 2001]. Methylglyoxal (MG) is one of physiological glucose metabolites [Thornalley, 1998; Kalapos, 1999]. A clinical study has shown that the median concentration of MG was 5–6-fold higher in blood samples from insulin-dependent diabetes mellitus (IDDM) patients and 2–3-fold higher in blood samples from non-insulin-dependent diabetes mellitus (NIDDM) patients than in control blood samples [McLellan et al., 1994]. MG that has accumulated abnormally in diabetes interacts strongly with and causes modification of cellular proteins and nucleic acids, resulting in cytotoxic events such as cell growth arrest, dysfunction, apoptosis, and

Grant sponsor: The Ministry of Education, Science, Sports and Culture of Japan.

*Correspondence to: Jun Du, Department of Medical Technology, Nagoya University School of Health Sciences, 1-1-20 Daikominami, Higashi-ku, Nagoya 461, Japan.
E-mail: togun@met.nagoya-u.ac.jp

Received 16 December 2002; Accepted 17 December 2002
DOI 10.1002/jcb.10478

necrosis, which are closely related to the development and progression of diabetes complications [Thornalley, 1996]. It is likely that MG elicits its adverse effects by altering various signal transduction pathways, which are used by cells to perform their functions and to maintain cellular integrity. We have recently found that MG provokes several signaling pathways and changes the intracellular redox state [Du et al., 2000a, 2001]. However, it has not been determined whether hyperglycemia and its metabolite, MG, affect growth factor-induced signal transduction systems and their cellular targets.

Human insulin-like growth factor-1 (IGF-I), one of the members of the insulin-like factor family, is a major mitogen in normal tissue and is likely to contribute significantly to the progression and growth of several cell types [Aaronson, 1991; Stewart and Rotwein, 1996]. It has been proposed that treatment of diabetes patients with IGF-I, as an adjunct to insulin, might delay or prevent progression of diabetic complications [Thrailkill, 2000]. IGF-I induces autophosphorylation of the IGF-I receptor and phosphorylation of its endogenous substrates, mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) [Kadowaki et al., 1987; Roth et al., 1992; Rosenzweig et al., 1994], events that appear to be critical for mediating signals leading to cell proliferation.

ERK has been shown to be activated under a high glucose condition [Haneda et al., 1997] and has been suggested to trigger cellular events necessary for the development of a number of diabetic complications such as diabetic neuropathy [Tomlinson, 1999]. Details of the mechanism of activation and the downstream signal cascade of ERK under a diabetes-induced condition remain, however, to be clarified. MG-induced cell growth arrest or apoptosis might play an important role in stimulating and accelerating diabetic complications, and treatment of diabetes with IGF-I might prevent and not worsen the development of diabetic complications. We have examined the effect of MG on IGF-I induced ERK-dependent cell growth response and analyzed the signal transduction cascade causing MG effects. The results showed that MG disrupted IGF-I-induced cell proliferation by enhancing MEKK1-dependent activation of ERK, which results in over-expression of $p21^{Waf1/Cip1}$.

MATERIALS AND METHODS

Antibodies and Chemical Reagents

Anti-MEK1, anti-ERK1, anti-$p21^{Waf1/Cip1}$, anti-His, and anti-MEKK1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-MEK1/2 and anti-phospho-ERK1/2 antibodies, PD98059 and LY294002, ATF2 peptide, and full length JNK1 protein were purchased from New England Biolabs, Inc. (Beverly, MA). GST-MKK4 protein was from Transduction Lab (Lexington, KY). MG and recombinant human IGF-I were from Sigma Chemical Co. (St. Louis, MO). Bisindolylmaleimide (BIM) was from CALBIOCEM (La Jolla, CA).

Plasmids

A murine MEK kinase 1 (MEKK1) fragment (amino acids 1,174–1,493) was prepared from the pCMV-MEKK1 vector (Clontech Laboratories, Inc., Palo Alto, CA) and was inserted into pUC118 plasmid at the SacI and kpnI sites. A point mutation in MEKK1, in which Lys1253 was changed to Met, was generated by PCR and subcloned in pcDNA4/HisMax (Invitrogen, San Diego, CA). The resulting plasmid was named MEKK1KM/His. pCMV-RafS621A was purchased from Clontech laboratories, Inc.

Cells Culture, Treatment, and Transfection

A human embryonic kidney cell line (HEK293) and mouse fibroblast cell line (NIH3T3) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in a CO_2 incubator. The cells were serum-deprived for 24 h prior to experimental manipulations. The cells were treated with PD98059 (40 μM), LY294002 (10 μM), or BIM (1 μM) for 1 h prior to incubation with IGF-I (100 ng/ml) or MG (1 mM for HEK293 cells and 0.25 mM for NIH3T3 cells). For transfection, cells were plated at a density of 1×10^4 cells/cm^2. After 24 h in culture, the cells were transfected with plasmid DNA (0.1 μg of DNA/cm^2 per plasmid) and FUGENE6 reagent according to the instructions of the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). Assays were performed at 36 h after transfection. At the end of all treatments, the cells were washed twice with ice-cold PBS and then lysed for whole cell lysate preparations.

Cell Lysis and Immunoblot Analysis

The immunoblot assays were performed as described previously [Du et al., 2000a]. Briefly, cells were lysed in lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 20 mg/ml aprotinin, and 5 mg/ml leupeptin. The lysates were cleared by centrifugation and denatured by boiling in Laemmli buffer, separated on 10 or 12% SDS–polyacrylamide gels (PAGE), and blotted onto a nitrocellulose membrane. Nonspecific binding was blocked by incubating the membrane with 0.05% Tween-20/PBS containing 5% non-fat dry milk for 1 h at room temperature. Membranes were incubated with the primary antibody overnight at 4°C and then with a horseradish peroxidase-conjugated secondary antibody, and the specific immune complexes were detected using Western Blot Plus Chemiluminescence Reagent (Life Science, Inc., Boston, MA).

MEKK1 Kinase Assay

To assess the MEKK1 kinase activity, the cells were lysed on ice as described above. The cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with MEKK1 antibody. The immunocomplexes were bound to protein A-Sepharose. The beads were washed twice with kinase buffer (20 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$) and subjected to the MEKK1 kinase assay. The immunocomplexes were incubated first with 0.1 μg of MKK4 for 15 min at 30°C in a final volume of 25 μl kinase buffer containing 100 μM of ATP and subsequently with 1 μg of JNK1 for 15 min at 30°C. Thereafter, the activated complex was incubated with 0.3 μCi of [γ-^{32}P]ATP and 1 μg of ATF2 peptide in the same solution. The samples were resolved by SDS–PAGE, and the phosphorylation of ATF2 was detected.

Cell Proliferation Assay

Cells were seeded in 96-well flat bottom culture plates at 5×10^3 cells per well and cultured in 100 μl of DMEM containing 10% FBS for 12 h and then incubated for an additional 24 h in a serum-free DMEM. The cells were stimulated with MG or IGF-I for 20 h. At 8 h before harvesting, ^{3}H-thymindine (37 kBq per well, Amersham-Japan Ltd., Tokyo, Japan) was added. After washing with PBS, cells were incubated with 50 μl of 0.25% trypsin at 37°C for 10 min, and then 150 μl of culture medium was added for deactivation of trypsin. The cells were then harvested onto filter paper, and the radioactivities were determined by liquid scintillation counting as described previously [Du et al., 2000b].

RESULTS

MG Inhibits IGF-I-Induced Cell Proliferation Through a MEK/ERK-Dependent Pathway

Both the HEK293 and NIH3T3 cells express functional IGF receptors and are known as excellent models for studying the regulatory mechanisms of cellular proliferation induced by growth factors. We used these two cell lines to examine the effects of MG on the cell proliferation stimulated by IGF-I. As shown in Figure 1, addition of MG alone did not change the level of DNA synthesis compared with the control basal level in the serum-free medium condition. Compared with the basal level, IGF-I increased the level of DNA synthesis by 2.5-fold in HEK293 cells and by seven fold in NIH3T3 cells. In the presence of MG, however, cell proliferation inducing effect of IGF-I was reduced, and the level of proliferation became lower than the background level. This inhibitory effect of MG on IGF-I-induced cell growth did not appear to be caused by promotion of cell death, because MG, at concentrations up to 3 mM (in HEK293 cells) and 1 mM (in NIH3T3 cells), did not induce a detectable level of apoptotic or necrotic cell death, as assessed by flowcytometry and the trypan blue dye exclusion test (data not shown). The MEK/ERK pathway, which is known to play a central role in IGF-I-mediated signaling for cell growth [LeRoith et al., 1995], may also be the target of the MG-mediated inhibition of the IGF-I effect. We examined the effect of a specific extracellular signal-regulated kinase (MEK) inhibitor, PD98059, on the MG effect. As also shown in Figure 1, addition of PD98059 partially prevented the MG-mediated inhibitory effect on the IGF-I-induced growth of both HEK293 and NIH3T3 cells. These study results were confirmed by additional experiments in which cell growth was evaluated directly by counting the number of cells (data not shown) and suggested that MG reversed the effect of IGF-I on cell proliferation, targeting MEK/ERK as potentially

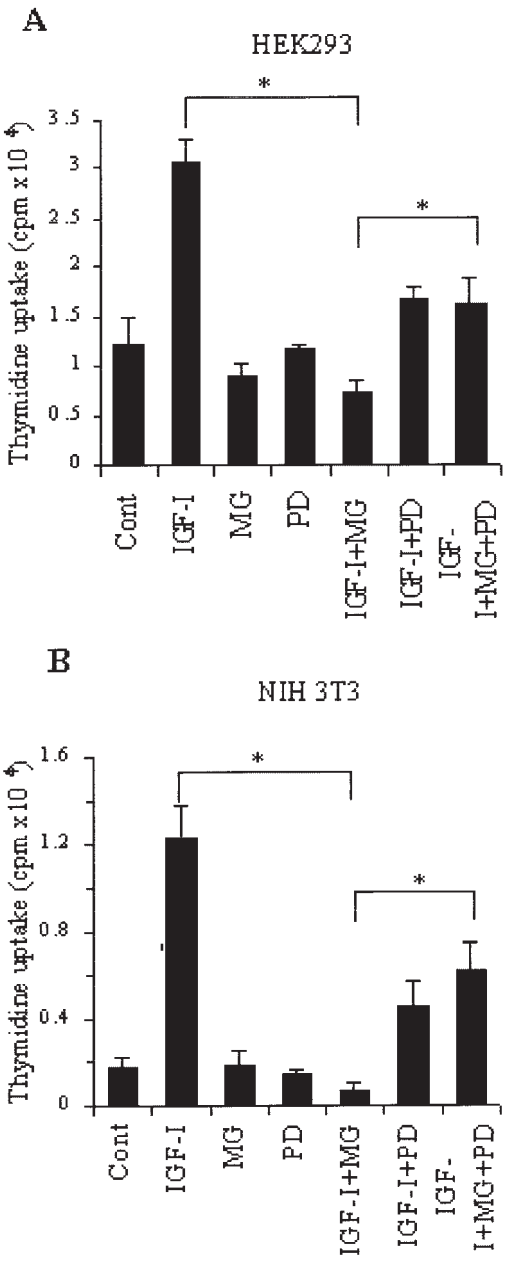

Fig. 1. Effect of MG on IGF-I-induced cell proliferation. HEK293 (**A**) and NIH3T3 (**B**) cells were cultured in serum-free medium for 24 h, followed by pretreatment with or without 40 μM PD98059 for 1 h, and then addition of IGF-I (100 ng/ml) or/ and MG (1 mM to HEK293 and 0.25 mM to NIH3T3 cells), and the cells were incubated for 20 h. During the last 8 h of incubation, ^{3}H-thymidine was added. After washing with PBS, cells were treated with trypsin, and then harvested onto filter paper. The radioactivity was determined by liquid scintillation counting. Mean counts per minute (cpm) from triplicate cultures are shown as representative results of three separate experiments. Statistically significant data are indicated by asterisks (*$P < 0.01$).

common elements in the signaling pathways triggered by IGF-I and MG.

MG Enhances IGF-I-Induced Activation of ERK

In order to determine the effect of MG on the activation of the MEK/ERK pathway induced by IGF-I, we examined the concentration- and time-dependent effects of MG on IGF-I-induced ERK activation. After being cultured in serum-free medium, HEK293 cells were pre-treated with MG for 1 h and then challenged with IGF-I for 5 min. The active (phosphorylated) forms of ERK and MEK were detected by immunoblotting with specific antibodies. As shown in Figure 2A, compared with a weak phosphorylation induced by MG alone, IGF-I caused obvious phosphorylation of ERK, which was further enhanced by pre-treatment with MG in a concentration-dependent manner. This synergistic effect of MG with IGF-I was most clearly observed at 1–2 mM of MG (Fig. 2A). Similar results were also obtained in NIH3T3 cells, which were more sensitive to pre-treatment with MG. Pretreatment of NIH3T3 cells with MG resulted in remarkable enhancement of IGF-I-induced activation of ERK. A maximal effect was observed at 0.25–0.5 mM of MG (Fig. 2B). Correspondingly, stimulatory effects of MG on IGF-I-induced phosphorylation of MEK were also observed in both cell lines (Fig. 2A,B). A time course study showed that when HEK293 cells were stimulated by IGF-I, ERK activity increased within 5 min, peaked at 5–10 min, and then declined to the basal level within 30 min (Fig. 2C). In the presence of MG, however, IGF-I induced a longer and more robust activation of ERK, which remained higher than the unstimulated initial levels for at least 2 h. The total protein amounts of ERK and MEK were basically unchanged during the entire periods of these experiments. In experiments of the same protocol, we also measured the kinase activity by an in vitro kinase assay on a substrate, myelin basic protein (MBP). Consistently with the results obtained for changes in phosphorylation level of ERK, treatment with MG and IGF-I together induced a longer and more robust increase in the kinase activity of ERK than did IGF-I alone (data not be shown). These results suggest that the MG-induced signal worked together with the IGF-I-mediated one for hyper-phosphorylation (hyper-activation) of MEK and ERK.

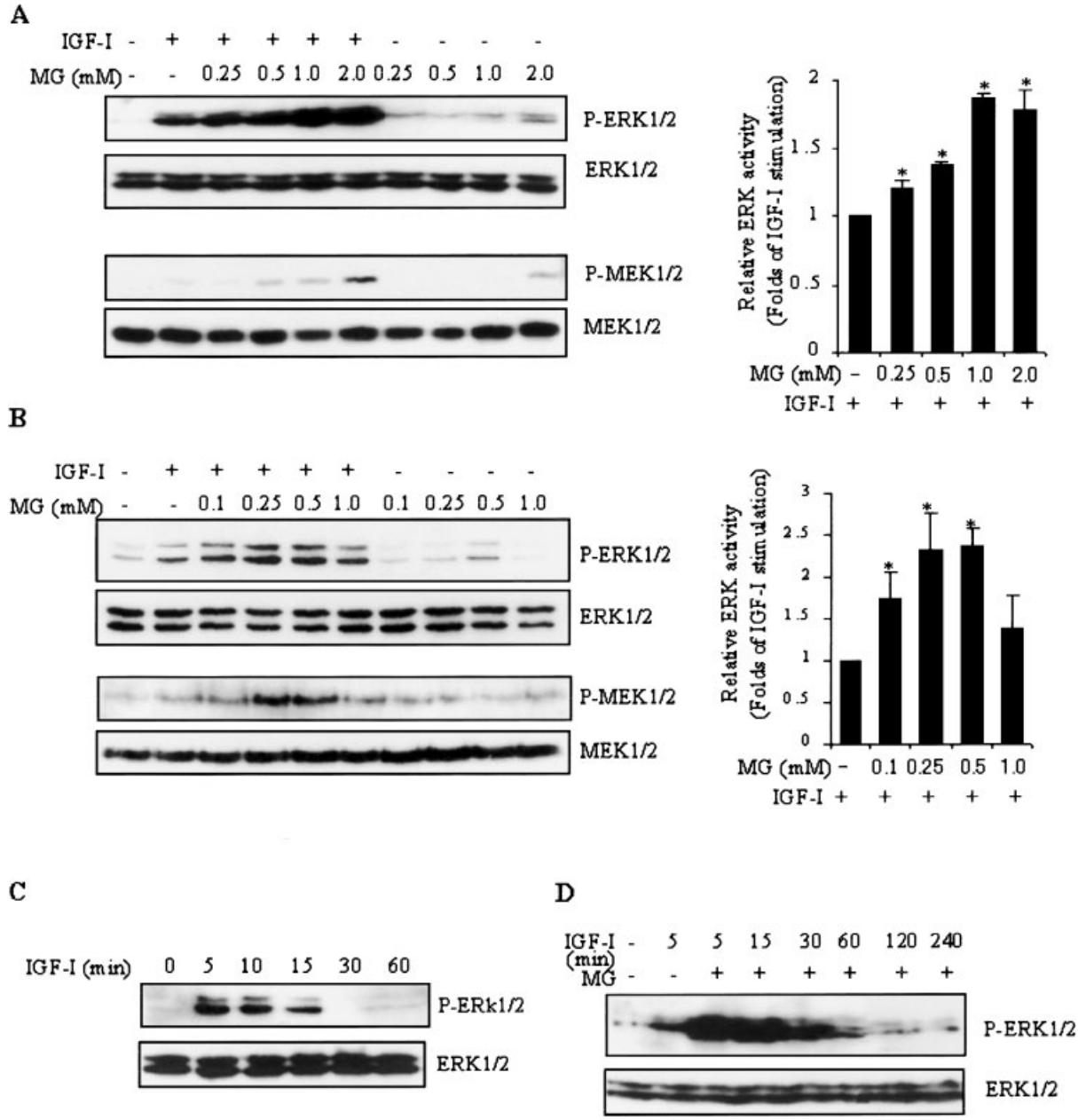

Fig. 2. Effect of MG on IGF-I-induced activation of MEK and ERK. Serum-starved HEK293 (**A**) and NIH3T3 (**B**) cells were pretreated with the indicated concentration of MG for 1 h and then stimulated with 100 ng/ml of IGF-I for 5 min. The cells were harvested and subjected to immunoblotting analysis with anti-phospho-MEK or anti-phospho-ERK antibodies. The membranes were stripped of antibodies and inmmunoblotted with anti-MEK and anti-ERK, respectively. The right parts of A and B show graphic representations of Scion Image analysis of phospho-ERK density from triplicate separate experiments. Fold indications of phospho-ERK as compared with the cells simulated by IGF-I alone are shown (data are mean ± SEM of three determinations, $^*P < 0.01$). Serum-starved HEK293 cells were treated with IGF-I for the indicated time (**C**) or pretreated or not pretreated with 1 mM MG for 1 h and then treated with 100 ng/ml of IGF-I for the indicated time (**D**). The phosphorylation level and the total protein amount of ERK were determined by immunoblotting with anti-phospho-ERK and anti-ERK1 antibodies, respectively. Similar results were obtained in three independent experiments.

MG-Mediated Enhancement of IGF-I-Induced ERK Activation Depends on MEK Activity but not on PI3K or PKC Activity

We next conducted experiments to determine the molecular mechanism of the synergy of MG with IGF-I in induction of hyper-activation of ERK. Earlier studies suggested that prolonged ERK activation could be achieved independently of MEK [Grammer and Blenis, 1997], while other studies suggested that phosphatidylinositol 3-kinase (PI3K) plays a role in ERK activation by regulating signaling events at the level of Ras [Yamauchi et al., 1993] or PKC [Liao et al., 1997]. In order to determine the roles of MEK, PI3K, and PKC in the enhancing effect of MG on IGF-I-induced ERK activation, their relevant specific inhibitors were used for potential inhibition. We found that pre-incubation of serum-starved HEK293 cells with PD98059 completely abolished not only ERK activation induced by IGF-I alone but also hyper-phosphorylation of ERK induced by co-stimulation with IGF-I and MG (Fig. 3A). However, no such inhibition was observed when cells were treated with a specific PI3K inhibitor, LY294002, at a concentration known to abolish PI3K activity (Fig. 3B). Consistent with this result, we also found that IGF-I-induced activation of Akt, an established downstream target of PI3K, was not enhanced by pre-treatment with MG (data not shown). A specific inhibitor of PKC, BIM, was also used to examine the role of PKC in the ERK hyper-activation caused by co-stimulation with IGF-I and MG. Figure 3B shows that BIM did not block the hyper-phosphorylation of ERK caused by co-stimulation with MG and IGF-I. These results suggest that MEK, rather than the PI3K or PKC pathway, is necessary for MG-mediated enhancement of IGF-I-induced ERK activation.

MEKK1 is an Important Component for MG-Mediated Enhancement of IGF-I-Induced ERK Activation

Raf-1 is a key protein in the transmission of and proliferation signals originating from receptor and non-receptor tyrosine kinases. To determine whether Raf-1 is involved in the enhancement of activation of the ERK pathway induced by co-stimulation with IGF-I and MG, HEK293 cells were transfected with a plasmid encoding the dominant-negative form of Raf-1 (Raf-1S621A). The extent of ERK phosphorylation was measured by immunoblotting. Figure 4A and B show that the action of IGF-I for inducing ERK phosphorylation in cells transfected with Raf-S621A was partially inhibited compared with that in cells transfected with an empty vector (Fig. 4B) but that the actions of MG for inducing ERK phosphorylation (Fig. 4A) or for enhancing IGF-I-induced ERK phosphorylation (Fig. 4B) were not diminished in the cells transfected with Raf-1S621A. These results suggested that MG enhanced IGF-I-induced phosphorylation of ERK through a Raf-1-independent signal transduction route.

The ability of MEKK1 to regulate sequential protein phosphorylation pathways, including those of MEK and ERK, has been demonstrated [Lange-Carter et al., 1993; Yan et al., 1994; Xu et al., 1996]. To determine the role of MEKK1 in hyper-phosphorylation of ERK caused by IGF-I and MG co-stimulation, we transfected HEK293 cells with a catalytically inactive form of MEKK1 (MEKK1KM) for 36 h and then serum-starved the cells for 18 h. In the cells

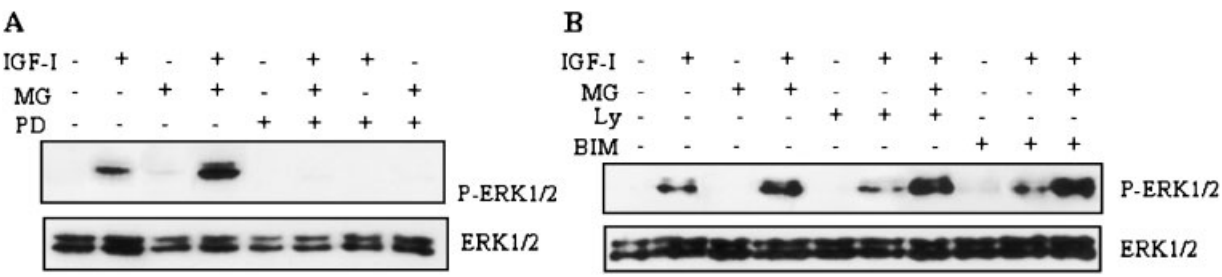

Fig. 3. Enhancement of IGF-I-induced ERK activation by MG is dependent on MEK activity but not on PI3K or PKC activity. Serum-starved HEK293 cells were pretreated with 40 μM of PD98059 (PD) (**A**), 10 μM of LY294002 (LY) (**B**), or 1 μM of BIM for 1 h, followed by addition of 1 mM of MG and further incubation for 1 h, and then treated with 100 ng/ml of IGF-I for 5 min. The phosphorylation level and the total protein amount of ERK were determined by immunoblotting with anti-phospho-ERK and anti-ERK antibodies, respectively. Similar results were obtained in three independent experiments.

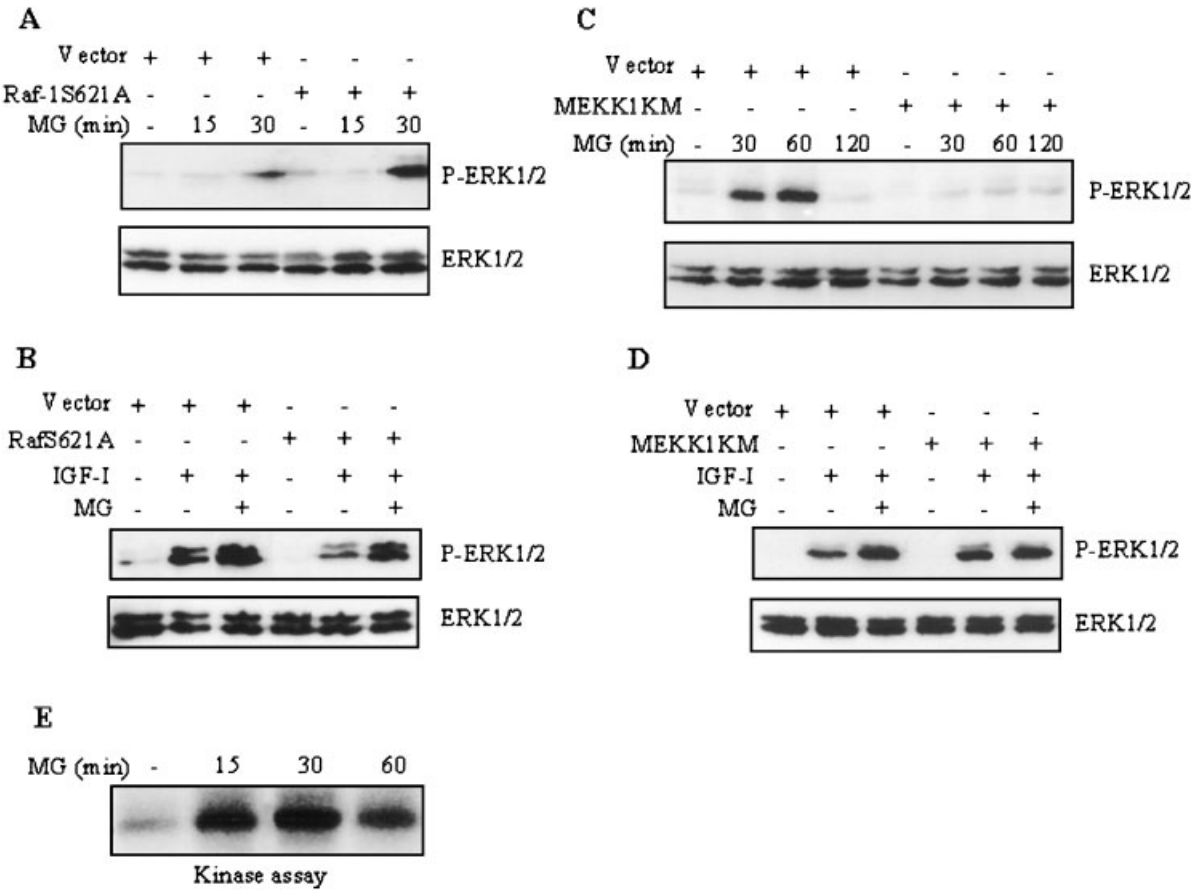

Fig. 4. MEKK1 is an important component for MG-mediated enhancement of IGF-induced ERK activation. HEK293 cells were transfected with an empty vector or a dominant negative form of Raf-1 (Raf-1S621A) (**A**, **B**) or a kinase-inactive form of MEKK1 (MEKK1KM) (**C**, **D**) for 24 h and then cultured in a serum-free medium for 18 h. The cells were treated with 2 mM MG for the indicated time (A, C) or pretreated with 1 mM MG for 1 h and then treated with 100 ng/ml of IGF-I for 5 min (B, D). The phosphorylation level and the total protein amount of ERK were determined by immunoblotting with anti-phospho-ERK and anti-ERK1 antibodies, respectively. After HEK293 cells had been incubated with or without 1.0 mM MG for 15–60 min, endogenous MEKK1 was immunoprecipitated with an anti-MEKK1 antibody, and its kinase activity was measured by a coupled kinase assay as described in Materials and Methods (**E**). Similar results were obtained in at least three independent experiments.

transfected with an empty vector, MG caused strong phosphorylation of ERK, which, however, was completely blocked by expression of MEKK1KM (Fig. 4C). Whereas the expression of the catalytically inactive form of MEKK1 never diminished, and even sometimes increased, the level of IGF-I-induced phosphorylation of ERK, the synergistic effect of co-stimulation with IGF-I and MG on ERK phosphorylation was almost abolished (Fig. 4D). Consistent with this result, we also found that treatment with MG rapidly induced activation of endogenous MEKK1 (Fig. 4E). These results suggested that MEKK1 was fundamentally involved in the MG-induced signal transduction pathway and the enhancement of IGF-I-dependent activation of MEK/ERK.

Synergy of MG With IGF-I for Hyper-Activation of MEK/ERK Results in Over-Expression of Endogenous $p21^{Waf1/Cip1}$

It has been shown that activation of ERK induces expression of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$, which functions as a universal inhibitor for the cell cycle and is able to inhibit the activities of several cyclins/CDKs for regulating the transition from G1 to S phases of the cell cycle, leading to cell growth arrest [Liu et al., 1996; Pumiglia and Decker, 1997; Kivinen and Laiho, 1999]. We next attempted to determine whether synergy of MG with IGF-I can promote the induction of $p21^{Waf1/Cip1}$ through hyper-activation of MEK/ERK in HEK293 and NIH3T3 cells. After being

pre-incubated for 1 h with or without PD98059, both serum-starved HEK293 and NIH3T3 cells were treated with IGF-I and/or MG for 8 h. The levels of $p21^{Waf1/Cip1}$ in cells were examined by immunoblotting with anti-$p21^{Waf1/Cip1}$ antibody. The results are shown in Figure 5A,B. Either IGF-I or MG alone resulted in a slight increase in the levels of the $p21^{Waf1/Cip1}$ compared with those in untreated quiescent cells. When cells were treated with IGF-I and MG together, the levels of $p21^{Waf1/Cip1}$ greatly increased in both HEK293 and NIH3T3 cells, and the levels were significantly higher than those induced by IGF-I or MG alone. This significant increase in the levels of $p21^{Waf1/Cip1}$ by a combination of IGF-I and MG was inhibited by PD98059. Furthermore, we investigated the role of kinase activity of MEKK1 in over-expression of $p21^{Waf1/Cip1}$ induced by MG and IGF-I co-stimulation. HEK293 cells were transfected with MEKK1KM and the levels of $p21^{Waf1/Cip1}$ were measured after incubation in the presence or absence of MG and IGF-I. As shown in Figure 5C, blocking of the kinase activity of MEKK1 by expression of MEKK1KM completely abolished the effect of co-stimulation with MG and IGF-I on $p21^{Waf1/Cip1}$ over-expression. These observations suggest that hyper-activation of MEK/ERK by a combination of MG and IGF-I contributes to cell growth arrest through over-expression of $p21^{Waf1/Cip1}$ and that kinase activity of MEKK1 is involved in this process.

DISCUSSION

Although the effect of stimulation with IGF-1 on proliferation or differentiation of cells has been extensively studied under physiologic conditions, it is still unclear whether IGF-1 can normally work in diabetes with hyperglycemia and abnormal accumulation of glucose metabolites such as MG. Some earlier studies showed that hyperglycemia disrupted the physiologic responses of cells to insulin and endothelin-1through activation of PKC isoforms and the ERK pathway [Glogowski et al., 1999; Kuboki et al., 2000]. In this study, we demonstrated that alteration of intracellular milieu of HEK293 or NIH3T3 cells by MG blocked cellular response to IGF-I for proliferation to IGF-1-induced proliferation through the unique synergy of MG with IGF-I in enhancement of phosphorylation of ERK, suggesting that alteration of physiologic signals transduced by hormone and growth factors might be one of important mechanisms by which MG promotes the development of chronic clinical complications in diabetics.

The cellular response to MG appears to be multiple and greatly dependent on the concentration of MG. At a high concentration, MG directly modifies protein amino acid residues, resulting in alterations of protein and receptor function, which have recently been implicated in the downregulation of epidermal growth factor receptor (EGFR) signaling [Portero-Otin et al., 2002]. Our previous studies demonstrated that a low concentration of MG induced phosphorylation of ERK by activating intracellular protein-tyrosine kinase (PTK), whereas a higher concentration downregulated ERK activity by aggregating cell surface and intracellular proteins [Akhand et al., 2001a,b]. In this study, we also found that, when the concentrations were higher than 1 mM (in HEK293 cells) and 0.25 mM (in HIN3T3 cells), the inhibition of IGF-I-induced cell growth by MG was not reversed by pretreatment with an MEK inhibitor (data not shown). However, at the concentration used in the present study, pretreatment of MG caused an increase in IGF-I-mediated ERK activation. This enhancement of the signaling event, which caused cell growth arrest, was reversible by the MEK inhibitor, suggesting that the inhibition of IGF-I-mediated cell growth by MG was due to alteration of intracellular signaling transduction events rather than impairment of the integrity of cells.

The MEK/ERK pathway has been shown to be activated by various stimuli, including stimulation by IGF-I, and to serve as one of key routes for IGF-I to carry out its multiple physio1ogical functions, such as cell proliferation and differentiation [LeRoith et al., 1995; Coolican et al., 1997]. However, some earlier studies demonstrated that the MEK/ERK pathway is also involved in cell proliferation inhibition induced by various stimuli. For example, ERK activation was shown to be critical for cell growth arrest in a human hepatocellular carcinoma cell line stimulated by hepatocyte growth factor [Tsukada et al., 2001], NIH 3T3 cells stimulated by nerve growth factor [Pumiglia and Decker, 1997], rat aortic smooth muscle cells stimulated by nitric oxide (NO) [Bauer et al., 2001], and the active form of Ras gene-transfacted cells [Olson

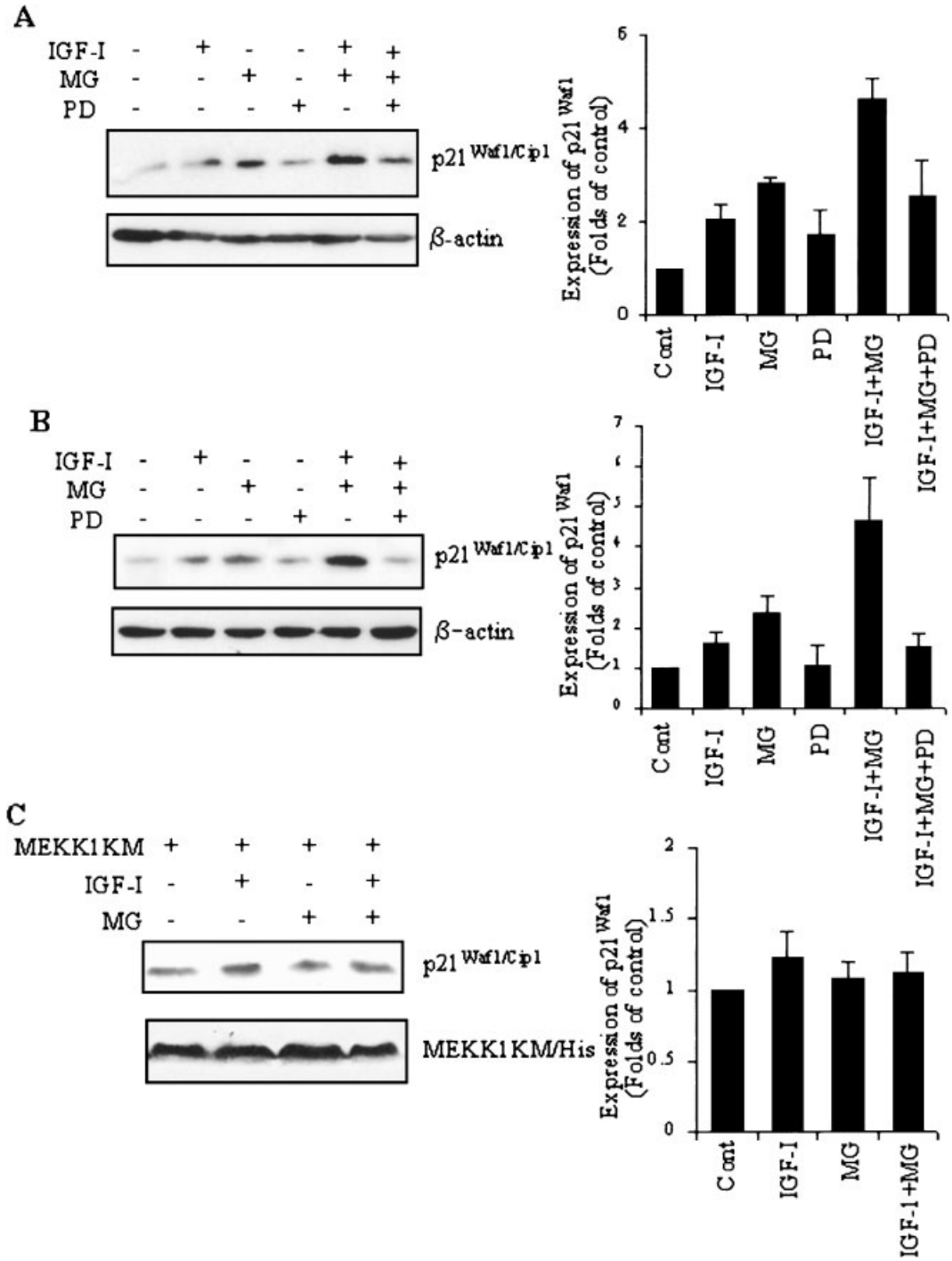

Fig. 5. MG synergizes with IGF-I to induce over-expression of $p21^{Waf1/Cip}$. Serum-starved HEK293 (**A**) and NIH3T3 (**B**) cells were pretreated with 40 μm PD98059 (PD) for 1 h, followed by addition of 1 mM (to HEK293 cells) or 0.25 mM (to NIH3T3 cells) MG and further incubation for 1 h. These cells were further treated with 100 ng/ml of IGF-I for 8 h. The expression levels of $p21^{Waf1/Cip}$ were determined by immunoblotting with an anti-$p21^{Waf1/Cip}$ antibody. The β-actin protein level was used as a loading control. **C**: HEK293 cells were transfected with MEKK1KM/His for 24 h and then cultured in a serum-free medium for 18 h. The cells were pretreated with 1 mM MG for 1 h and then treated with 100 ng/ml of IGF-I for 8 h. The expression level of $p21^{Waf1/Cip1}$ was determined by immunoblotting with an anti-$p21^{Waf1/Cip1}$ antibody (**upper panel**). The **lower panel** shows the expression level of MEKK1KM/His. The panels on the right show graphic representations of $p21^{Waf1/Cip1}$ density analyzed by Scion Image program (Scion Corporation, Frederick, MD) from three separate experiments. Fold expression of $p21^{Waf1/Cip1}$ as compared with unstimulated cells is shown.

et al., 1998]. The cell proliferation inhibition in these experiments was always accompanied by a strong and prolonged activation of the ERK pathway, and this cell growth arrest could be restored by inhibition of ERK pathway activity. In contrast, treatment of cells with mitogenic stimuli, which leads to cell proliferation, causes a transient, perhaps biphasic, increase in ERK activity [Waskiewicz and Cooper, 1995]. These findings suggest that the level of signaling activity of ERK has a range suitable for leading to proliferation stimulation and that proliferation inhibition is induced when ERK signaling activity exceeds this range. A possible mechanism of cell proliferation inhibition by strong activation of the ERK pathway is up-regulation of $p21^{Waf1/Cip1}$ expression, which induces cell cycle arrest by inhibition of CDK activity [Harper et al., 1993; Pumiglia and Decker, 1997]. In this study, we found that MG caused significant suppression of IGF-I-induced cell proliferation in close association with a marked enhancement of ERK activation and increase in the level of $p21^{Waf1/Cip1}$ expression, both of which were prevented by a MEK1/2 inhibitor, PD98059, demonstrating the requirement of activation of ERK for the induction of $p21^{Waf1/Cip1}$ and cell proliferationinhibition. Although IGF-I-induced cell proliferation is known to be mediated by activation of cyclin D-CDK4/6 and cyclin E-CDK2 [Nevins, 1998; Sherr and Roberts, 1999], these cell proliferation regulatory molecules have been shown to be targets of $p21^{Waf1/Cip1}$ for cell proliferation inhibition [Harper et al., 1993]. Thus, our results suggest that MG and IGF-I synergistically act on the MEK/ERK pathway for hyper-activation, which in turn causes induction of $p21^{Waf1/Cip1}$ for inhibition of IGF-I-induced cell growth.

What could be the molecular mechanism of the synergy of MG with IGF-I for induction of hyper-activation of ERK? It is known that multiple signals can activate ERK signaling through a variety of pathways. We demonstrated from the results of experiments showing high sensitivity of MEK to the specific inhibitor PD98059 that the pathway for MG/IGF-I-mediated hyper-activation of ERK involves MEK. A number of earlier studies showed that PI3K and PKC, which were activated by growth factors, including IGF-I [Myers et al., 1992; Skolnik et al., 1993], activated the MEK/ERK signaling pathway through activation of Ras and Raf-1 [Yamauchi et al., 1993; King et al., 1997; Chaudhary et al., 2000]. Pre-incubation of the cells with LY294002, a specific inhibitor of PI3K, or BIM, a specific inhibitor of PKC, did not, however, block hyper-phosphorylation of ERK induced by combined treatment with MG and IGF-I. These results indicated that neither PI3K nor PKC was involved in the signaling for hyper-activation of ERK trigged by MG and IGF-I. Among the many signaling elements for ERK activation, Raf-1 is an important kinase and is sufficient to activate the MEK/ERK pathway in cells responding to many exogenous stimuli. Our results show that the expression of a dominant negative form of Raf-1 reduced IGF-I-induced ERK activation in the absence of MG but had no obvious effect of ERK phosphorylation by MG alone or MG-mediated enhancement of IGF-I-induced ERK activation, suggesting that Raf-1 is not involved in the signaling for the MG-induced ERK activation.

MEKK1 is an important mediator of multiple stress-activated signaling pathways and can be activated in various stress responses, including a change in the cellular redox state [Schaeffer and Weber, 1999; Widmann et al., 1999; Davis, 2000]. In fact, our previous study showed that addition of MG to Jurkat cells resulted in rapid generation of intracellular reactive oxygen species (ROS), which in turn triggered activation of ASK1, a MAP kinase kinase kinase related to MEKK1, and JNK [Du et al., 2001]. Thus, we speculated that ROS generated by MG treatment contribute to the activation of MEKK1, which in turn activates MEK and ERK. In this study, we found that MEKK1 was rapidly activated by treatment with MG. Moreover, we noticed that the expression of a catalytically inactive form of MEKK1 but not that of Raf-1S621A strongly suppressed MG-induced activation of ERK, suggesting that MEKK1, rather than Raf-1, is fundamentally involved in the MG-induced signal transduction pathway. The blockade of MEKK1 activation by expression of a kinase inactive form of MEKK1 had no effect on IGF-I-induced phosphorylation of ERK but reduced the synergistic effect of co-stimulation with IGF-I and MG on ERK phosphorylation. These results support the view that MG and IGF-I work in different ways to activate the MEK/ERK pathway; i.e., Raf-1 and MEKK1 activities are preferentially required for IGF-I- and MG-mediated signaling, respectively.

In summary, this study has demonstrated that alteration of intracellular milieu induced by MG results in the modification of cell response to IGF-I. The counter action of MG on IGF-I-induced cell proliferation was found to be mediated through activation of the MEKK1 pathway, which, acting as an upstream signal molecule, enhances the IGF-I-mediated activation of ERK and promotes expression of $p21^{Waf1/Cip1}$. These results suggest a new role of MG in the development of chronic complications of diabetes.

REFERENCES

Aaronson S. 1991. Growth factors and cancer. Science 254: 1146–1153.

Akhand AA, Hossain K, Mitsui H, Kato M, Miyata T, Inagi R, Du J, Takeda K, Kawamoto Y, Suzuki H, Kurokawa K, Nakashima I. 2001a. Glyoxal and methylglyoxal trigger distinct signals for MAP family kinases and caspase activation in human endothelial cells. Free Radic Biol Med 31:20–30.

Akhand AA, Hossain K, Kato M, Miyata T, Du J, Suzuki H, Kurokawa K, Nakashima I. 2001b. Glyoxal and methylglyoxal induce glyoxal and methyglyoxal induce aggregation and inactivation of ERK in human endothelial cells. Free Radic Biol Med 31:1228–1235.

Bauer PM, Buga GM, Ignarro LJ. 2001. Role of p42/p44 mitogen-activated-protein kinase and p21waf1/cip1 in the regulation of vascular smooth muscle cell proliferation by nitric oxide. Proc Natl Acad Sci USA 98:12802–12807.

Brownlee M. 2001. Biochemistry and molecular cell biology of diabetic complications. Nature 414:813–820.

Chaudhary A, King WG, Mattaliano MD, Frost JA, Diaz B, Morrison DK, Cobb MH, Marshall MS, Brugge JS. 2000. Phosphatidylinositol 3-kinase regulates Raf1 through Pak phosphorylation of serine 338. Curr Biol 10:551–554.

Coolican SA, Samuel DS, Ewton DZ, McWade FJ, Florini JR. 1997. The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. J Biol Chem 272:6653–6662.

Davis RJ. 2000. Signal transduction by the JNK group of MAP kinases. Cell 103:239–252.

Du J, Suzuki H, Nagase F, Akhand AA, Yokoyama T, Miyata T, Kurokawa K, Nakashima I. 2000a. Methylglyoxal induces apoptosis in Jurkat leukemia T cells by activating c-Jun N-terminal kinase. J Cell Biochem 77: 333–344.

Du J, Suzuki H, Nagase F, Akhand AA, Yokoyama T, Nakashima I. 2000b. Mercuric chloride stimulates distinct signal transduction pathway for DNA synthesis in a T-cell line, CTLL-2. J Cell Biochem 78:500–508.

Du J, Suzuki H, Nagase F, Akhand AA, Ma XY, Yokoyama T, Miyata T, Nakashima I. 2001. Superoxide-mediated early oxidation and activation of ASK1 are important for initiating methylglyoxal-induced apoptosis process. Free Radic Biol Med 31:469–478.

Glogowski EA, Tsiani E, Zhou X, Fantus IG, Whiteside C. 1999. High glucose alters the response of mesangial cell protein kinase C isoforms to endothelin-1. Kidney Int 55:486–499.

Grammer TC, Blenis J. 1997. Evidence for MEK-independent pathways regulating the prolonged activation of the ERK-MAP kinases. Oncogene 14:1635–1642.

Haneda M, Araki S, Togawa M, Sugimoto T, Isono M, Kikkawa R. 1997. Mitogen-activated protein kinase cascade is activated in glomeruli of diabetic rats and glomerular mesangial cells cultured under high glucose conditions. Diabetes 46:847–853.

Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75:805–816.

Kadowaki T, Koyasu S, Nishida E, Tobe K, Izumi T, Takaku F, Sakai H, Yahara I, Kasuga M. 1987. Tyrosine phosphorylation of common and specific sets of cellular proteins rapidly induced by insulin, insulin-like growth factor I, and epidermal growth factor in an intact cell. J Biol Chem 262:7342–7350.

Kalapos MP. 1999. Methylglyoxal in living organisms: Chemistry, biochemistry, toxicology, and biological implications. Toxicol Lett 110:145–175.

King WG, Mattaliano MD, Chan TO, Tsichlis PN, Brugge JS. 1997. Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. Mol Cell Biol 17: 4406–4418.

Kivinen L, Laiho M. 1999. Ras- and mitogen-activated protein kinase kinase-dependent and -independent pathways in $p21^{Waf1/Cip1}$ induction by fibroblast growth factor-2, platelet-derived growth factor, and transforming growth factor-beta1. Cell Growth Differ 10: 621–628.

Kuboki K, Jiang ZY, Takahara N, Ha SW, Igarashi M, Yamauchi T, Feener EP, Herbert TP, Rhodes CJ, King GL. 2000. Regulation of endothelial constitutive nitric oxide synthase gene expression in endothelial cells and in vivo: A specific vascular action of insulin. Circulation 101:676–681.

Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ, Johnson GL. 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science 260:315–319.

LeRoith D, Werner H, Beitner-Johnson D, Roberts CT, Jr. 1995. Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocr Rev 16:143–163.

Liao DF, Monia B, Dean N, Berk BC. 1997. Protein kinase C-zeta mediates angiotensin II activation of ERK1/2 in vascular smooth muscle cells. J Biol Chem 272:6146–6150.

Liu Y, Martindale JL, Gorospe M, Holbrook NJ. 1996. Regulation of $p21^{Waf1/Cip1}$ expression through mitogen-activated protein kinase signaling pathway. Cancer Res 56:31–35.

McLellan AC, Thornalley PJ, Benn J, Sonksen PH. 1994. Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. Clin Sci (Lond) 87:21–29.

Myers MG, Jr., Backer JM, Sun XJ, Shoelson S, Hu P, Schlessinger J, Yoakim M, Schaffhausen B, White MF. 1992. IRS-1 activates phosphatidylinositol 3′-kinase by

associating with src homology 2 domains of p85. Proc Natl Acad Sci USA 89:10350–10354.

Nevins JR. 19918. Toward an understanding of the functional complexity of the E2F and retinoblastoma families. Cell Growth Differ 9:585–593.

Olson MF, Paterson HF, Marshall CJ. 1998. Signals from Ras and Rho GTPases interact to regulate expression of p21Waf1/Cip1. Nature 394:295–299.

Portero-Otin M, Pamplona R, Bellmunt MJ, Ruiz MC, Prat J, Salvayre R, Negre-Salvayre A. 2002. Advanced glycation end product precursors impair epidermal growth factor receptor signaling. Diabetes 51:1535–1542.

Pumiglia KM, Decker SJ. 1997. Cell cycle arrest mediated by the MEK/mitogen-activated protein kinase pathway. Proc Natl Acad Sci USA 94:448–452.

Rosenzweig SA, Oemar BS, Law NM, Shankavaram UT, Miller BS. 1994. Insulin like growth factor 1 receptor signal transduction to the nucleus. In: LeRoith D, Raizada MK, editors. Current directions in insulin-like growth factor research. New York: Plenum Press. p 159–168.

Roth RA, Zhang B, Chin JE, Kovacina K. 1992. Substrates and signaling complexes: The tortured path to insulin action. J Biol Chem 48:12–18.

Schaeffer HJ, Weber MJ. 1999. Mitogen-activated protein kinases: Specific messages from ubiquitous messengers. Mol Cell Biol 19:2435–2444.

Sherr CJ, Roberts JM. 1999. CDK inhibitors: Positive and negative regulators of G1-phase progression. Genes Dev 13:1501–1512.

Skolnik EY, Lee CH, Batzer A, Vicentini LM, Zhou M, Daly R, Myers MJ, Jr., Backer JM, Ullrich A, White MF, Schlessinger J. 1993. The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and Shc: Implications for insulin control of ras signaling. EMBO J 12:1929–1936.

Stewart CE, Rotwein P. 1996. Growth, differentiation, and survival: Multiple physiological functions for insulin-like growth factors. Physiol Rev 76:1005–1026.

Thornalley PJ. 1996. Pharmacology of methylglyoxal: Formation, modification of proteins and nucleic acids, and enzymatic detoxification—a role in pathogenesis and antiproliferative chemotherapy. Gen Pharmacol 27:565–573.

Thornalley PJ. 1998. Glutathione-dependent detoxification of alpha-oxoaldehydes by the glyoxalase system: Involvement in disease mechanisms and antiproliferative activity of glyoxalase I inhibitors. Chem Biol Interact 111–112:137–151.

Thrailkill KM. 2000. Insulin-like growth factor-I in diabetes mellitus: Its physiology, metabolic effects, and potential clinical utility. Diabetes Technol Ther 2:69–80.

Tomlinson DR. 1999. Mitogen-activated protein kinases as glucose transducers for diabetic complications. Diabetologia 42:1271–1281.

Trial Research Group. 1993. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 329:977–986.

Tsukada Y, Miyazawa K, Kitamura N. 2001. High intensity ERK signal mediates hepatocyte growth factor-induced proliferation inhibition of the human hepatocellular carcinoma cell line HepG2. J Biol Chem 276:40968–40976.

UK Prospective Diabetes Study (UKPDS) Group. 1998. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). Lancet 352:837–853.

Waskiewicz AJ, Cooper JA. 1995. Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. Curr Opin Cell Biol 7:798–805.

Widmann C, Gibson S, Jarpe MB, Johnson GL. 1999. Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human. Physiol Rev 79:143–180.

Xu S, Robbins DJ, Christerson LB, English JM, Vanderbilt CA, Cobb MH. 1996. Cloning of rat MEK kinase 1 cDNA reveals an endogenous membrane-associated 195-kDa protein with a large regulatory domain. Proc Natl Acad Sci USA 93:5291–5295.

Yamauchi K, Holt K, Pessin JE. 1993. Phosphatidylinositol 3-kinase functions upstream of Ras and Raf in mediating insulin stimulation of c-fos transcription. J Biol Chem 268:14597–14600.

Yan M, Dal T, Deak JC, Kyriakis JM, Zon LI, Woodgett JR, Templeton DJ. 1994. Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. Nature 372:798–800.