Selective Induction of Heparin-binding Epidermal Growth Factor-like Growth Factor by Methylglyoxal and 3-Deoxyglucosone in Rat Aortic Smooth Muscle Cells

THE INVOLVEMENT OF REACTIVE OXYGEN SPECIES FORMATION AND A POSSIBLE IMPLICATION FOR AtherosGENESIS IN DIABETES

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Methylglyoxal (MG) and 3-deoxyglucosone (3-DG), reactive dicarbonyl metabolites in the glyoxalase system and glycation reaction, respectively, selectively induced heparin-binding epidermal growth factor (HB-EGF)-like growth factor mRNA in a dose- and time-dependent manner in rat aortic smooth muscle cells (RASMC). A nuclear run-on assay revealed that the dicarbonyl may regulate expression of HB-EGF at the transcription level. The dicarbonyl also increased the secretion of HB-EGF from RASMC. However, platelet-derived growth factor, another known growth factor of smooth muscle cells (SMC), was not induced by both dicarbonyls. The dicarbonyl increased intracellular peroxides prior to the induction of HB-EGF mRNA as judged by flow cytometric analysis using 2',7'-dichlorofluorescin diacetate. N-Acetyl-L-cysteine and aminoguanidine suppressed both dicarbonyl-induced HB-EGF mRNA and intracellular peroxide levels in RASMC. DL-Buthionine-(S,R)-sulfoximine increased the levels of 3-DG-induced HB-EGF mRNA. Furthermore, hydrogen peroxide alone also induced HB-EGF mRNA in RASMC. These results indicate that MG and 3-DG induce HB-EGF by increasing the intracellular peroxide levels. In addition, the pretreatment with 12-O-tetradecanoylphorbol-13-acetate failed to alter dicarbonyl-induced HB-EGF mRNA expression in RASMC, suggesting that the signal transducing mechanism is not mediated by protein kinase C. Since HB-EGF is known as a potent mitogen for smooth muscle cells and is abundant in atherosclerotic plaques, the induction of HB-EGF by MG and 3-DG, as well as the concomitant increment of intracellular peroxides, may trigger atherogenesis during diabetes.

Methylglyoxal (2-oxopropanal; MG), a reactive α,β-dicarbonyl metabolite and physiological substrate for the glyoxalase system (1), is formed by the non-enzymatic and enzymatic elimination of phosphate from dihydroxyacetone phosphate, glyceraldehyde-3-phosphate (2, 3), and by the oxidation of hydroxyacetone and aminocetone (4–6). The estimated rate of formation of methylglyoxal in tissues of normal healthy subjects is approximately 125 μg/day which can largely be accounted for as a result of fragmentation of triose phosphates (2). The glyoxalase system, using reduced glutathione as a cofactor, catalyzes the conversion of methylglyoxal to α-lactate via the intermediate β-lactylglutathione. The formation of methylglyoxal in cultured human red blood cells is increased under hyperglycemic conditions and by the addition of fructose, α-glyceroldehyde, dihydroxyacetone, acetone, and hydroxyacetone (7, 8). The serum concentration of methylglyoxal increases 5–6-fold in patients with insulin-dependent diabetes mellitus and 2–3-fold in patients with non-insulin-dependent diabetic mellitus (9).

3-Deoxyglucosone (3-DG), another dicarbonyl, is a major and highly reactive intermediate in the glycation reaction and a potent cross-linker responsible for the polymerization of proteins to form advanced glycation end products (10). Plasma 3-DG levels are also increased under diabetic conditions (11). In our previous studies, the enzyme that reduces 3-DG was identified as an aldehyde reductase (12). A preliminary study indicated that 3-DG induced HB-EGF in RASMC (13). Biochemical and clinical evidence suggests that the increased formation of MG or 3-DG in diabetes mellitus is linked to the development of diabetic complications, but the exact role of these dicarbonyls in this process remains largely unknown.

Proliferation of vascular smooth muscle cells represents a crucial event in the development of atherosclerotic lesions (14–17). These cells migrate from the media to the intima of the aorta and proliferate in response to growth factors, such as platelet-derived growth factor (PDGF) (14–16, 18). Our previous study indicated that in the case of vascular smooth muscle cells of diabetic rats, an increased mitogenic response was observed for heparin-binding epidermal growth factor-like growth factor (HB-EGF), a member of the EGF family (19). Recent studies showed that significant amounts of HB-EGF are produced in SMC and macrophages of atherosclerotic plaques (20). This suggests that HB-EGF could be implicated in atherogenesis. Other growth regulators, such as basic fibroblast growth factor, insulin-like growth factor-1, transforming growth factor-β, IL-1, and tumor necrosis factor-α, also appear to be involved in atherogenesis (14, 17).
The aim of the present study was to examine the role of MG and 3-DG in the regulation of the expression of growth factors in relation to the development of diabetic complications. The data suggest that MG and 3-DG selectively induce HB-EGF in primary rat aortic smooth muscle cells (RASMC) by increasing the level of intracellular peroxides. This may provide a new insight into the mechanism by which diabetic complications, such as atherosclerosis, arise.

**EXPERIMENTAL PROCEDURES**

**Materials**—Methylglyoxal was purchased from Sigma and further purified by distillation under reduced pressure (b.p. 26 °C, 20 mm Hg) and the purity was confirmed by NMR spectroscopy. The concentration of MG in stock solutions was determined by an end point enzymatic assay involving conversion to 5-D-lactoylglutathione with glyoxalase I (Sigma) and hydrolysis catalyzed by glyoxalase II (Sigma) (21). 

107 Bq/mmol (specific activity of 980 dpm/nmol) was a generous gift of Dr. Paul J. Thornalley (University of Essex) and was diluted with unlabeled MG and used at a concentration of 160 μM and specific activity of 980 dpm/nmol. 3-DG was chemically synthesized according to Khadem and et al. (22). Anti-rat HB-EGF neutralizing antibody number 19 (1 mg/ml) was a generous gift from Judy Abraham (Scios). 

Aminoguanidine, cycloheximide, and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from the Wako Pure Chemical Industry. 2′, 7′-Dichlorofluorescin diacetate (H2DCF-DA) was from Molecular Probes, Inc. Other chemicals were of the highest grade available.

**Cell Culture**—RASMC were isolated from the thoracic aorta of a Wistar rat (body weight 200 g) as described previously (23) and cultured in Dulbecco’s modified Eagle’s medium (Nikken Bio Med Lab) with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5% CO2 at 37 °C. The cells were passaged every 4–6 days. RASMC were cultured to about 80% confluence and further incubated with fresh medium containing the above reagents. Cell viabilities were measured by trypan blue exclusion after incubation with reagents. Throughout these experiments, the cells were used within passages 4–8.

**RNA Isolation and Northern Blot Analysis**—After incubation, as described under “Results,” RASMC were washed twice with ice-cold phosphate-buffered saline and the total RNA was extracted with acid guanidium thiocyanate-phenol-chloroform as reported previously (25). 20 μg of the total RNA were run on a 1% agarose gel containing 2.2 mol/liter formaldehyde. The size-fractionated RNAs were transferred onto Zeta-Probe membranes (Bio-Rad) overnight by capillary action. Rat HB-EGF cDNA (5′ EcoRI-PstI linker) (26), human PDGF A chain cDNA (EcoRI linker), and human PDGF B chain cDNA (EcoRI linker) (27) were labeled with [α-32P]dCTP (NEN Life Sciences Products) using random primers and unlabeled dCTP (Multiprimer DNA labeling system, Pharmacia). After hybridization with the labeled probes at 42 °C in the presence of 50% formamide, the membrane was washed twice with 2 × sodium chloride-sodium citrate (SSC; 1 × SSC, 15 mmol/liter sodium citrate 150 mmol/liter NaCl, pH 7.5) which contained 0.1% sodium dodecyl sulfate at 50 °C for 60 min, and then washed with 0.2 × SSC and 0.1% SDS at 50 °C for 30 min. Kodak X-AR films were exposed for 1–2 days to an intensifying screen at −80 °C. The intensities of bands on x-ray films were quantitated with a CS-9000 gel scanner (Shimadzu, Japan).

**Transcriptional Analysis**—A nuclear run-on assay was conducted as described (28) with slight modifications. After RASMC (approximately 5 × 106 cells/dish) were stimulated with MG for 3 h or with 3-DG for 1.5 h in the presence or absence of actinomycin D, nuclei were prepared from the cells with 1 ml of Nonidet P-40 lysis buffer. Each nuclear run-on reaction mixture (400 μl) contained 200 μl of nuclei, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, and 150 μCi of [γ-32P]UTP (>81 GBq/mmol, Amersharm Corp.). This mixture was incubated for 30 min at 30 °C, followed by digestion with DNase I and proteanase K, and by extraction with phenol/chloroform/isoamyl alcohol. An equal amount of radiolabeled RNA was suspended at >5 × 106 cpm/ml in a hybridization buffer. 10 μg of the unlabeled linearized plasmid cDNA probes for hybridization to run-on products were dot blotted onto Nylon membranes. The membranes were hybridized as described for Northern blotting.

**Soluble Growth Factor Assay**—EP170.7 cells, which are 32D cells stably expressing the EGF receptor and which proliferate in response to IL-3 or EGF receptor ligand, were washed three times and then resus-...
ice-cold phosphate-buffered saline, the intracellular peroxide levels were measured using a FACScan (Becton Dickinson, Mountain View, CA). For image analysis, cells were analyzed for fluorescence intensity using a lysis cell analysis system (30).

**Statistical Analysis**—Data were analyzed by the Student’s t test and the results were expressed as means ± S.D.

**RESULTS**

**Effects of MG and 3-DG on HB-EGF and PDGF mRNA Expression Levels in RASMC**—The effect of MG and 3-DG on transcriptional levels of HB-EGF and PDGF in RASMC was examined by Northern blot analysis. When RASMC were incubated with 0–400 μM MG for 6 h, a significant, dose-dependent increase was observed in HB-EGF mRNA levels, although the low level of HB-EGF mRNA was detected before treatment as reported previously (26) (Fig. 1A). The levels of HB-EGF mRNA were increased 3–6.5-fold, in response to treatment with 100–400 μM MG. After treatment with 0.5–10 mM 3-DG for 2 h, the levels of HB-EGF mRNA increased 0.5–3-fold (Fig. 1B). However, MG and 3-DG had no effect on PDGF-A and PDGF-B mRNA levels under these conditions. These results demonstrate that MG and 3-DG are capable of selectively inducing HB-EGF mRNA in RASMC.

**Time Courses for HB-EGF mRNA Induction and HB-EGF Secretion by MG and 3-DG**—MG and 3-DG induced HB-EGF mRNA in a time-dependent manner. After addition of MG, the levels of HB-EGF mRNA increased at 3 h and reached a maximum at 6 h for the case of RASMC. The elevated HB-EGF mRNA levels returned to the near base line after 12 h (Fig. 2A). On the other hand, the level of HB-EGF mRNA increased at 1 h after treatment with 3-DG. The elevated HB-EGF mRNA levels by 3-DG increased at 1 h, reached a maximum at 2 h and returned to the near base line after 5 h (Fig. 2B).

To know MG and 3-DG have additive effects on HB-EGF mRNA levels, a time course study was carried out. We found that 3-DG slightly increased MG-induced HB-EGF mRNA levels at 6 h (data not shown), indicating that metabolite(s) of 3-DG may be involved in this event.

Induction of mRNA in response to a stimulus is not always followed by the translation of the corresponding protein. It was therefore important to demonstrate whether MG and 3-DG also could induce HB-EGF at the protein level. HB-EGF is synthesized as a membrane-anchored precursor that can be processed to a soluble form after proteolytic cleavage (31). Therefore, to examine the secreted growth factor activity in conditioned media from MG or 3-DG-treated RASMC, we performed a bioassay which estimates mitogenic activity for EP170.7 cells that require IL-3 or EGF receptor ligand for cell growth (24). After RASMC were incubated with or without MG or 3-DG for various times, the conditioned media were collected. In the absence of IL-3, EP170.7 cells were incubated with the conditioned media, and the uptake of [3H]thymidine into DNA was measured (C). Values plotted are the means of triplicate determinations. Similar results were obtained in four independent experiments. * and ** denote p < 0.01 and p < 0.05, respectively.
creased after 15 min, while the radioactivity of MG in the conditioned media was decreased by 11%. The MG incorporated into the cells was 1.8% of the total MG, suggesting that some MG still remained on the cell surface and the small portion incorporated into the cells may be involved in the induction of HB-EGF mRNA.

Effects of Cycloheximide, Actinomycin D, and Aminoguanidine on the Enhancement of HB-EGF mRNA Levels by MG or 3-DG—We evaluated the effects of aminoguanidine, a scavenger of dicarbonyl and an inhibitor of glycation (32, 33), on the enhancement of HB-EGF mRNA by MG and 3-DG. As shown in Fig. 5, 1 mM aminoguanidine blocked both MG and 3-DG-induced HB-EGF expressions in RASMC. This suggests that MG or 3-DG may directly induce HB-EGF mRNA in RASMC. Induction of HB-EGF mRNA during treatment with the dicarbonyl was also completely abolished by an inhibitor of RNA synthesis, actinomycin D. Moreover, MG had no effect on the stability of HB-EGF mRNA in the presence of actinomycin D (data not shown). To examine the effect of the dicarbonyl on transcription of the HB-EGF gene, a nuclear run-on analysis was also performed. Both MG and 3-DG enhanced the transcription of HB-EGF gene (Fig. 6), whereas actinomycin D inhibited the stimulated transcription of HB-EGF gene. Thus, MG or 3-DG stimulated accumulation of HB-EGF mRNA is a result of the activation of gene transcription.

Cycloheximide did not block the levels of MG and 3-DG-increased HB-EGF mRNA, but rather induced the expression of HB-EGF mRNA (data not shown) as reported previously (34). This may be due to a superinduction of HB-EGF by cycloheximide. These results indicate that the induction of HB-EGF mRNA by MG and 3-DG does not require the synthesis of new proteins.

Involvement of Reactive Oxygen Species in MG or 3-DG-induced HB-EGF mRNA Expression—It has been reported that MG produces reactive oxygen species (ROS) (35, 36), which may contribute to the induction of HB-EGF gene. MG and 3-DG caused a significant increase in intracellular peroxide levels as early as 1 h, prior to the induction of HB-EGF mRNA level in RASMC (Fig. 7A). 3-DG also caused a significant increase in intracellular peroxide levels at 30 min (Fig. 7B). Aminoguanidine blocked the formation of intracellular peroxides by MG. Moreover, the increased intracellular peroxides were completely suppressed by preincubation with 10 mM N-acetyl-L-cysteine (Fig. 7C), which is capable of causing an increase in intracellular GSH levels thus resulting in the scavenging of oxidants both directly and indirectly (37–39). After treatments with aminoguanidine or NAC and 3-DG, similar results were observed in intracellular peroxide production (data not shown). These results indicate that MG and 3-DG cause a rapid increase in the levels of intracellular peroxides, which appear to be involved in the induction of the HB-EGF gene in RASMC.

To investigate the role of ROS during induction of HB-EGF...
by MG and 3-DG, the effect of NAC on MG or 3-DG-induced HB-EGF mRNA level in RASMC was examined. The effect of BSO, a reagent which depletes intracellular GSH levels, on these inductions was also examined. The cells were incubated with 10 mM NAC or 10 mM BSO for 24 h, and the cells were then treated with MG or 3-DG for an additional 6 or 2 h, respectively. BSO pretreatment failed to alter MG-induced HB-EGF mRNA, but increased the levels of 3-DG-induced HB-EGF mRNA, and NAC pretreatment completely abolished both MG and 3-DG-increased gene expression level (Fig. 8). We also found that NAC suppressed the levels of MG-induced HB-EGF mRNA in a dose-dependent manner (data not shown). These results, therefore, indicate that the dicarboxyl-induced peroxides are involved in the induction of HB-EGF in RASMC. Based on these results, a reasonable assumption is that hydrogen peroxide or other peroxides participate in the regulation of the HB-EGF gene in RASMC. Thus, we examined the effect of hydrogen peroxide on the expression levels of HB-EGF mRNA. The incubation of RASMC with hydrogen peroxide also increased the levels of HB-EGF mRNA in a dose- and time-dependent manner in RASMC (Fig. 9). This finding also strongly supports the hypothesis that ROS are involved in induction of HB-EGF. Taken together, the present study indicates that MG and 3-DG induce the expression of HB-EGF mRNA by increasing ROS in RASMC.

**Effect of TPA Pretreatment on HB-EGF mRNA Induction by MG and 3-DG—TPA, a specific protein kinase C (PKC) activator, can rapidly and potently stimulate cells to produce ROS (40). On the other hand, TPA also induces HB-EGF mRNA in SMC (26). These findings prompted us to examine whether MG induces HB-EGF mRNA, by activating PKC to produce intracellular peroxides. RASMC were preincubated with 50 nM TPA for 24 h, during which protein kinase C is down-regulated by persistent treatment with TPA (41). The cells were further incubated with 400 μM MG for 6 h or 5 mM 3-DG or with 50 nM TPA for 30 min. TPA pretreatment and down-regulation of protein kinase C suppressed TPA-induced HB-EGF mRNA (data not shown), but had no effect on the accumulation of HB-EGF mRNA by MG or 3-DG (Fig. 10). These results suggest that induction of the HB-EGF gene by dicarbonyls in RASMC is independent of protein kinase C activity.

**DISCUSSION**

Diabetes mellitus is commonly accompanied by atherosclerosis. SMC proliferation plays an important role in the development of atherosclerotic lesions (14–17). Interestingly, it has been reported that among the members of the EGF family only HB-EGF appears to be involved in atherogenesis so far. The present study was undertaken in an attempt to evaluate the effect of MG and 3-DG on expression levels of HB-EGF and PDGF in RASMC and to explore the mechanism to this.

We have shown that MG and 3-DG significantly induce HB-EGF and that the induced HB-EGF mRNA levels are due to activation of this gene transcription, which is not dependent upon de novo protein synthesis. MG and 3-DG, however, had no effect on PDGF-A and PDGF-B mRNA levels under the same conditions. These results suggest that MG and 3-DG may be involved in a specific signal pathway and thereby selectively induce HB-EGF. Although the concentration of MG needed to induce the HB-EGF in these studies was in the order of 100–400 μM and appears to be slightly higher than concentrations in diabetes, 5–10 μM. The concentrations of MG as judged by the 14C-methylglyoxal incorporation was much lower (in the range of 2–4 μM), suggesting that MG probably as well as 3-DG may act at physiologically relevant concentrations. It is possible that lower concentration of dicarbonyl, as seen in patients with diabetes, would be sufficient to induce HB-EGF mRNA in the cells.

ROS have been implicated in the etiology of a large number of diseases, such as cancer, inflammation, aging, and diabetic complications (42, 43). MG rapidly generates ROS including superoxides, during the glycation reaction (36), increases ROS in hepatocytes (35). MG and 3-DG also induce apoptotic cell death in macrophage-derived cell lines (44). Chronic exposure to abnormally high concentrations of these dicarbonyl, therefore, may be a contributing factor in oxidative stress in diabetes mellitus. The present study showed that, prior to the induction of HB-EGF mRNA, MG significantly increased intracellular peroxides in RASMC (Fig. 7A). There are several possible ways by which intracellular ROS levels could be increased by MG. One is that ROS are produced during the glycation reaction of amino acids or proteins with MG. Another is that the glutathione content of cells are depleted during MG metabolism by the
mRNAs. The fact that H2O2 increased the level of HB-EGF suggests that oxidative stress is involved in MG and 3-DG-induced HB-EGF mRNA in RASMC.

Our study indicates that oxidative stress may also contribute to the growth of vascular smooth muscle cells but not endothelial cells or fibroblasts (49, 50). A previous report indicates that ROS specifically stimulates the growth of vascular smooth muscle cells but not endothelial cells or fibroblasts.

ROS activate cellular growth and play a role in the regulation of intracellular signals, such as intracellular alkalinization, lipid peroxidation, and ultimately cell lysis. Several studies indicate that the ROS can also stimulate growth-related intracellular signals, such as intracellular alkalinization (47) as well as increase in c-fos and c-myc proto-oncogene mRNA levels (48). ROS activate cellular growth and play a significant role in tumor promotion and degenerative diseases (49, 50).

ROS stimulate the growth of vascular smooth muscle cells but not endothelial cells or fibroblasts (51). Our study indicates that oxidative stress is involved in MG and 3-DG-induced HB-EGF mRNA in RASMC. The fact that H2O2 increased the level of HB-EGF mRNA and an antioxidant, NAC, completely abolished both the dicarbonyl-induced ROS and HB-EGF mRNA in RASMC supports these conclusions. These results provide strong evidence that MG and 3-DG trigger HB-EGF mRNA induction in RASMC by creating a state of oxidative stress.

NF-κB activation is known to be regulated by ROS (52). Thus, we also examined whether NF-κB is activated by MG and 3-DG using a gel mobility shift assay in conjunction with two types of the mouse NF-κB probe (53). After incubation with MG or 3-DG, the extent of NF-κB activation was not measurably changed, contrary to our expectations (data not shown). Therefore, NF-κB does not appear to be a responsive factor for the dicarbonyl-induced HB-EGF.

Another transcription factor, AP-1, appears to respond to ROS, as well (54). ROS activates c-Jun NH2-terminal kinases (JNK) (55) and the activated JNK phosphorylates c-Jun (56), leading to stimulation of AP-1 transcriptional activity. There is an AP-1 consensus sequence in the HB-EGF promoter (53, 57). JNK and the AP-1 sites may play an important role in the dicarbonyl-induced HB-EGF gene expression and further study will be necessary to elucidate the role of the JNK signaling pathway and the AP-1 site in the induction of HB-EGF by the dicarbonyl.

Both MG and 3-DG-induced ROS generation and HB-EGF mRNA increases were also suppressed by aminoguanidine, which is an efficient scavenger of dicarbonyl, as well as an inhibitor of glycation. Aminoguanidine can suppress the formation of the dicarbonyl-modified proteins (32, 33). These inhibitory effects of aminoguanidine may be related to the proposed potential efficacy in the prevention and therapy of diabetic complications.

Activated protein kinase C participates in the production of ROS (40) and the induction of HB-EGF mRNA (26). However, while ROS directly increased DNA synthesis and cell numbers in SMC, this effect was independent of protein kinase C activation (49). The present finding showed that pretreatments of RASMC with 50 nM TPA for 24 h, which down-regulates protein kinase C, had no effect on the dicarbonyl-induced HB-EGF mRNA levels, indicating that the dicarbonyl-induced HB-EGF mRNA in RASMC is independent from protein kinase C activity.

In our study, we also found that MG and 3-DG induced HB-EGF mRNA transiently and the dicarbonyl-increased ROS did not maintain for a long time (data not shown). This is because the dicarbonyl is unstable and metabolized very easily both in RASMC and in the culture medium. Regular stimulation of RASMC by the dicarbonyl during diabetes may produce ROS and induce HB-EGF permanently, which result in initiation of smooth muscle proliferation in atherosclerosis. It is also likely that the dicarbonyl-increased HB-EGF affects some other kinds of growth factors, cytokines, and vasoregulatory molecules which may participate in this process.

To our knowledge, this is the first demonstration that MG and 3-DG induce HB-EGF by provoking oxidative stress. MG and 3-DG, through these effects, may be involved in the development of diabetic complications such as atherosclerosis.
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