Advanced Glycation End Product Precursors Impair Epidermal Growth Factor Receptor Signaling

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Formation of advanced glycation end products (AGEs) is considered a potential link between hyperglycemia and chronic diabetic complications, including disturbances in cell signaling. It was hypothesized that AGEs alter cell signaling by interfering with growth factor receptors. Therefore, we studied the effects of two AGE precursors, glyoxal (GO) and methylglyoxal (MGO), on the epidermal growth factor receptor (EGFR) signaling pathway in cultured cells. Both compounds prevented tyrosine autophosphorylation induced by epidermal growth factor (EGF) in a time- and dose-dependent manner as well as phospholipase Cγ1 recruitment and subsequent activation of extracellular signal-regulated kinases. AGE precursors inhibit EGF-induced EGFR autophosphorylation and tyrosine kinase activity in cell membranes and in EGFR immunoprecipitates. In addition, AGE precursors strongly inhibited cellular phosphotyrosine phosphatase activities and residual EGFR dephosphorylation. AGE precursors induced the formation of EGFR cross-links, as shown by the cross-reactivity of modified EGFR with an anti-N$^{\epsilon}$(carboxymethyl) lysine antibody, suggesting that altered EGFR signaling was related to carbonyl-amine reactions on EGFR. Aminoguanidine, an inhibitor of AGE formation, partially prevented the EGFR dysfunction induced by GO and MGO. These data introduce a novel mechanism for impaired cellular homeostasis in situations that lead to increased production of these reactive aldehydes, such as diabetes. *Diabetes* 51:1535–1542, 2002

The Maillard reaction comprises the nonenzymatic reaction of reducing sugars with molecules containing an amino group, including proteins, phospholipids, and DNA (1,2). After the reversible formation of a Schiff's base between the reducing sugar and free amino group, this reaction proceeds to form advanced glycation end products (AGEs). Aldehydes such as glyoxal (GO) and methylglyoxal (MGO) are also able to induce AGE formation (3). GO and MGO can be generated during glycation of proteins by glucose (3); during lipid peroxidation (4); from metabolic processes, such as base-catalyzed phosphate elimination of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate; and also during the metabolism of acetone and threonine (5).

During chronic complications of diabetes and aging, the levels of AGEs derived from the reaction of GO and MGO with proteins rise in plasma proteins, extracellular matrix, and lens proteins (6–8). Modification of proteins through derivatization by GO and MGO may induce interaction with specific receptors, such as the RAGE and AGE-R family (9), or directly alter protein functions. For instance, MGO inhibits mitochondrial respiration, membrane ATPases and glyceraldehyde-3-phosphate dehydrogenases (10), and DNA and protein synthesis, thereby inducing growth arrest and cell death (11).

Because altered response to growth factor may be implicated in the pathogenesis of diabetic ulcers (12) and vascular diseases associated with diabetes (13), we hypothesized that GO and MGO may modify growth factor receptors and alter the subsequent signaling. Our investigations were mainly focused on the epidermal growth factor receptor (EGFR) because high glucose concentrations lead to abnormal epidermal growth factor (EGF) signaling (14) and diabetes reduces EGFR autophosphorylation (15–17).

The EGFR belongs to a large family of tyrosine kinase receptors and is involved in the regulation of multiple cellular processes, such as cell growth, motility, differentiation, survival, and death. The EGFR is a 170-kDa transmembrane receptor tyrosine kinase that is shared by several growth factors, including EGF, heparin-binding EGF, transforming growth factor-α, amphiregulin, neuroregulin, β-cellulin, and epiregulin (18). Ligand binding induces EGFR dimerization, stimulation of intrinsic tyrosine kinase, and autophosphorylation of its own tyrosine residues. These residues are binding sites for SH2 domains of enzymatic proteins, such as phospholipase Cγ1 (PLCγ1), phosphatidylinositol 3-kinase, phosphotyrosine phosphatase SHP2, *ras*-GTPase activating protein, and/or adaptors such as Shc isoforms, Grb2, Grb7, and Nck, which mediate downstream signaling (rev. in 18).

In the present study, we report that the AGE precursors GO and MGO alter EGF-induced EGFR activation in

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AGE, advanced glycation end product; CML, N$^{\epsilon}$(carboxymethyl)lysine; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellularly regulated kinase; GO, glyoxal; [^{3}H]SP, [^{3}H]succinimidyl propionate; MGO, methylglyoxal; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PLCγ1, phospholipase Cγ1; PTPase, protein tyrosine phosphatase; PVDF, polyvinylidene difluoride.

cultured cells through a mechanism related to AGE formation in EGFR.

RESEARCH DESIGN AND METHODS

Chemicals. RPMI-1640 containing 2 mmol/l Glutamax, Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and FCS were purchased from Gibco (Cergy-Pontoise, France). [^{3}H]succinimidyl propionate ([^{3}H]SP), horseradish peroxidase–conjugated sheep anti–mouse Ig, and the ECL^r (enhanced chemiluminescence) system were obtained from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). [γ-^{33}P]ATP (5 Ci/mmol) was supplied by ICN Biomedicals (Orsay, France). Anti-phosphotyrosine (4G10, mouse monoclonal) was purchased from UBI (Euromedex, Souffelweyersheim, France), anti-EGFR (rabbit polyclonal) and anti–platelet-derived growth factor receptor (PDGFR) were from Santa Cruz (Tebu, France), and horseradish peroxidase–conjugated goat anti–rabbit and acrylamide bisacrylamide were from Bio-Rad (München, Germany). Polyvinylidene difluoride (PVDF) Immobilon-P membranes came from Millipore (Bedford, MA) and human recombinant EGF and platelet-derived growth factor (PDGF) from PeproTech (Tebu, France). GO, MGO, and protein G-Sepharose were from Sigma (St. Louis, MO). Anti-phosphorylated (activated) extracellularly regulated kinases (ERKs), and anti–total ERK antibodies were supplied by Promega (Madison, WI). Rabbit polyclonal anti-N$^\epsilon$(carboxymethyl)lysine (CML) antiserum was obtained as previously described (19).

Cell culture, dicarbonyl treatments, and viability assessment. ECV304, A431 cell lines, and smooth muscle cells were obtained from the American *Type Culture* Collection (Rockville, MD). The EGFR-transformed fibroblast cell line B82-K+, overexpressing human EGFR, was generously donated by Dr. M.J. Weber (Health Sciences Center, Department of Microbiology, University of Virginia, Charlottesville, VA) (20).

Under standard conditions, cells were grown in RPMI-1640 (ECV304) or in DMEM (A431 and B82K+) supplemented with 10% heat-inactivated FCS, Glutamax, 100 units/ml penicillin, and 100 μg/ml streptomycin, as previously described (21). All passages were made at a split ratio of 1:3.

Cell viability was evaluated by using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test (21) and the trypan blue exclusion test. Subconfluent cells were starved overnight in serum-free RPMI before addition of GO or MGO at the times and concentrations indicated below. EGFR was stimulated by adding 10 nmol/l EGF at 37°C for 15 min. EGF pulse-chase experiments were performed as previously described (22).

Western blot and immunoprecipitation analyses. Cells were washed in ice-cold PBS containing 20 mmol/l NaF, 20 mmol/l $Na_4P_2O_7$, 1 mmol/l Na_3VO_4, and 5 mmol/l EDTA. Cells were lysed with solubilizing buffer (50 mmol/l HEPES [pH 7.4], 150 mmol/l NaCl, 1 mmol/l Na_3VO_4, 20 mmol/l NaF, 10 mmol/l $Na_4P_2O_7$, 2.5 mmol/l phenylmethylsulfonyl fluoride, 10 μmol/l leupeptin, 10 μg/ml aprotinin, 10% glycerol, 1% Triton X-100, and 0.5% sodium deoxycholate) for 30 min on ice. Insoluble materials were removed by centrifugation (15,000 rpm at 4°C for 15 min).

Cell proteins (50 μg) or, when indicated, immunoprecipitated EGFR were resolved by SDS-PAGE electrophoresis, transferred onto PVDF membranes that were blocked with 1% BSA, probed with the appropriate primary antibody, and revealed by a peroxidase-coupled secondary antibody and ECL detection, as previously indicated (23). The detection of CML adducts was performed using anti-CML antiserum as previously described (19). ERK activation was investigated through Western blot experiments on cell homogenates using an anti-phosphorylated ERK (anti-pERK) antibody and an anti-total ERK (anti-ERK) antibody.

In vitro EGFR autophosphorylation and tyrosine kinase activity. EGFR autophosphorylation and tyrosine kinase activity were assayed on EGFR immunoprecipitated from ECV304 cells or on plasma membranes isolated from A431 cells (overexpressing EGFR), under the previously used conditions (23). Briefly, after preincubation with GO or MGO, EGFR immunoprecipitates or A431 plasma membranes (40 μg/assay) were incubated in phosphorylation buffer (50 mmol/l HEPES [pH 7.5], 150 mmol/l NaCl, 10 mmol/l $MnCl_2$, 10 mmol/l $MgCl_2$, 10 μmol/l Na_3VO_4, and 0.02% Triton X-100) containing 60 μmol/l ATP in a final volume of 25 μl. After stimulation by EGF (10 nmol/l at 37°C for 15 min), the reaction was stopped by adding 15 μl of 4 × sample-reducing buffer (19) and heating at 90°C for 5 min. Tyrosine phosphorylation and EGFR content were analyzed by SDS-PAGE and Western blot as described above.

Alternatively, autophosphorylation of EGFR was studied by adding 5 μCi of [γ-^{33}P]ATP (3,000 Ci/mmol) to the phosphorylation buffer. The reaction was stopped by spotting an aliquot of the mixture on phosphocellulose membranes, and its radioactivity was subsequently counted (23). Tyrosine kinase activity was evaluated in the same conditions by phosphorylation of poly-Glu-Tyr (23).

Derivatization of the EGFR free amino groups. After preincubation of ECV304 cells with 5 mmol/l GO or MGO for 5 h, EGFR was immunoprecipitated, and the free amino group content was evaluated by using the amine-reactive probe [^{3}H]SP (23). Free amino groups were derivatized with 10 μCi of [^{3}H]SP in borate buffer 0.5 mol/l, pH 8.5, at 4°C for 15 min. The immunoprecipitates were washed three times in borate buffer and boiled in 4 × sample-reducing buffer. EGFR were then resolved by SDS-PAGE, the 170-kDa bands were recovered, and the radioactivity was counted by liquid scintillation (23).

Determination of protein tyrosine phosphatase activity. Protein tyrosine phosphatase (PTPase) activity was determined according to Buscail et al. (24). Briefly, PTPase activity of homogenates of cells (preincubated with GO and then stimulated by EGF) was evaluated by [^{33}P]poly-Glu-Tyr dephosphorylation under the previously used conditions (25). Protein concentrations were measured by the bicinchoninic acid method (23).

RESULTS

GO and MGO inhibit EGFR tyrosine phosphorylation and subsequent cell signaling. In ECV304 preincubated with GO or MGO for increasing periods of time, EGF-induced phosphorylation of cell proteins was progressively inhibited in a time-dependent manner, and the inhibitory effect of GO was obvious after 2-h preincubation (or 30 min in the case of MGO) (Fig. 1*A* and *B*). As expected, the 170-kDa band phosphorylated after EGF stimulation was identified as EGFR by immunoprecipitation followed by Western blot, as shown in Fig. 2.

The dose-response study showed that under the conditions used, the inhibitory effect of the EGF-induced EGFR autophosphorylation began at 0.5 mmol/l GO or MGO and was higher at 5 mmol/l (Fig. 1*C*). This inhibitory effect was not restricted to the ECV304 cell line but was observed on all the cell lines used in this study, namely A-431 cancer cells, B82K+ fibroblasts, and rabbit vascular smooth muscle cells.

It may be noted that at the concentrations used here and over the course of the experiments (<7 h), we observed no loss of cell number and of cell viability (as assessed by MTT test and trypan blue exclusion test). When cells were incubated with GO or MGO for longer incubation times, a moderate toxic effect was observed after 24-h incubation with 5 mmol/l GO or MGO but not with 0.5 mmol/l GO or MGO (data not shown). This suggests that the inhibition of EGF-induced EGFR autophosphorylation resulting from the 6-h preincubation with 0.5 or 5 mmol/l GO or MGO cannot be attributed to cell loss or a toxic effect at that time.

To evaluate whether inhibition by GO and MGO of the EGF-induced EGFR autophosphorylation was associated with alteration of the downstream signaling, we examined some of the mediators of this signaling pathway. As shown by coimmunoprecipitation experiments (Fig. 2*A*), preincubation of cells with GO inhibited the recruitment of PLCγ1 to EGF-activated EGFR. In addition, the EGF-induced activation of the serine-threonine kinases ERK1/2, other downstream effectors of growth factor signaling pathways, were also inhibited by GO and MGO (Fig. 2*B* and *C*). Interestingly, with MGO, ERK1/2 inactivation was more rapid than that of EGFR, thus suggesting that an additional mechanism may be involved in the MGO-induced inactivation of ERKs, in agreement with the recent report of Akhand et al. (26).

The inhibition elicited by GO and MGO on the response of these effectors (PLCγ1 and ERK1/2) is consistent

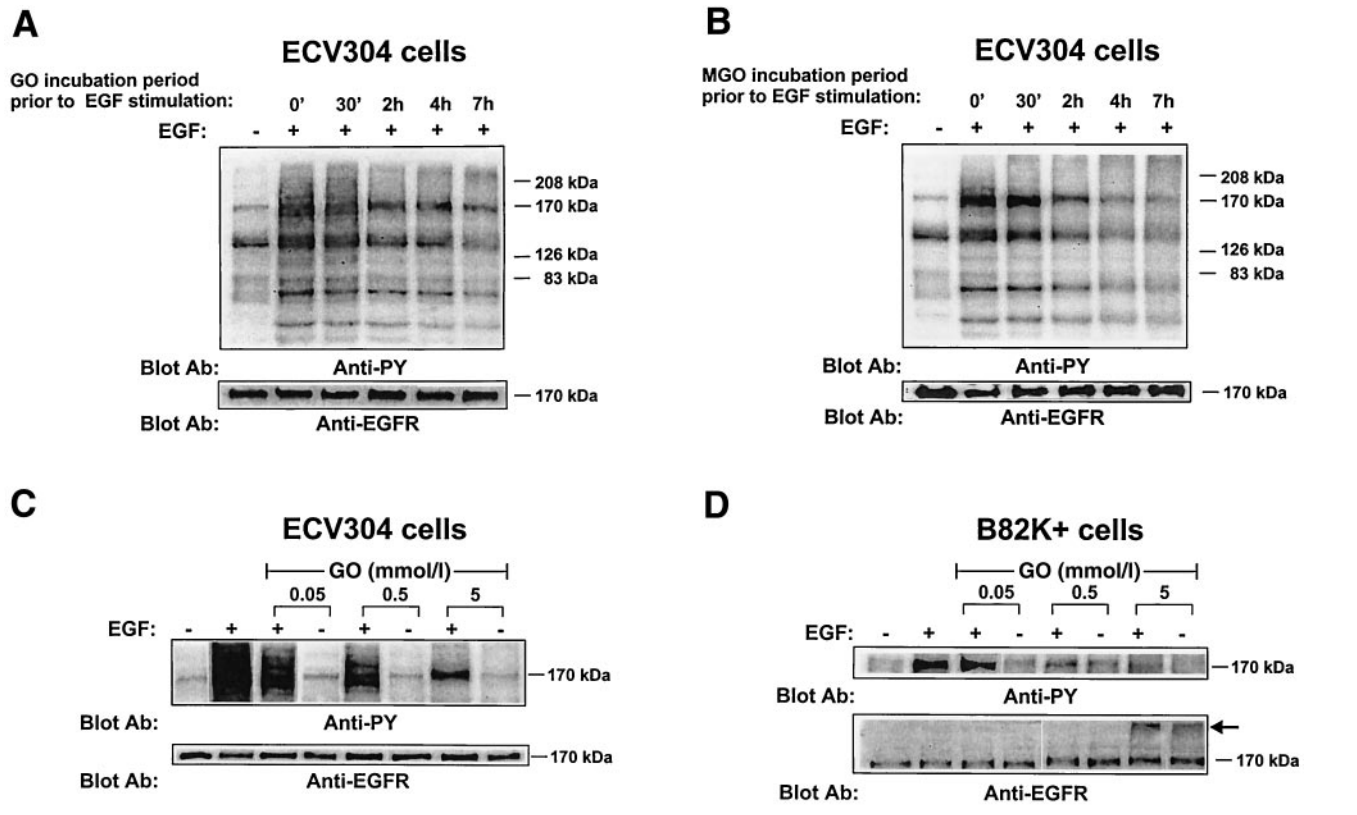

FIG. 1. EGFR tyrosine phosphorylation is inhibited by GO and MGO. Cells were starved for 16 h in RPMI-1640 containing 0.5% FCS and then incubated with GO or MGO at the indicated times and concentrations. Cell lysates were analyzed by Western blotting (SDS-PAGE on 7.5% polyacrylamide gels) and probed with anti-phosphotyrosine (anti-PY) or anti-EGFR. Data shown are representative of four experiments. *A* and *B*: Time course of inhibition of EGF-induced EGFR autophosphorylation on ECV304 cells, either control or incubated with 5 mmol/l GO (*A*) or MGO (*B*) for the indicated time and stimulated by EGF (10 nmol/l for 15 min). *C* and *D*: Dose response of EGFR tyrosine phosphorylation in ECV304 cells (*C*) and B82K+ fibroblasts (*D*) incubated (or not) for 6 h with GO at the indicated concentrations and stimulated by EGF (10 nmol/l for 15 min). The arrow indicates the presence of anti-EGFR reactive high-molecular-weight aggregates.

with the inhibitory effect of these aldehydes on the EGF-induced EGFR activation.

Mechanisms involved in GO- or MGO-induced EGFR inhibition

The inhibitory effect did not result from EGF modification by GO and MGO. The inhibitory effect was probably not due to a direct effect of GO and MGO on EGF because the inhibitory effect was dependent on the time of contact of GO/MGO with cells (in the absence of EGF) and was independent of the time of contact of GO/MGO with EGF (15-min incubation) (Fig. 1*A* and *B*) and because the same inhibitory effect was observed in experiments with continuous presence of GO/MGO (i.e., when EGF was added to the medium containing GO/MGO at the end of preincubation) and in pulse-chase experiments (i.e., when EGF was added after washing out GO/MGO at the end of the 6-h preincubation, just before addition of EGF) (data not shown).

The inhibitory effect did not result from a loss of EGFR protein induced by GO and MGO. The inhibitory effect did not result from a loss of EGFR protein because when the same amount of cell protein extract was used for Western blots revealed by anti-EGFR, a similar level of EGFR protein was detected (Figs. 1 and 2) and because when the same amount of immunoprecipitated EGFR protein was used for Western blots revealed by anti-phosphotyrosine and anti-EGFR, the EGFR tyrosine phosphorylation (i.e., reactivity with anti-phosphotyrosine) was reduced, whereas the amount of EGFR protein (i.e., reactivity with anti-EGFR) remained unchanged (Figs. 1 and 2). This strongly suggests that moderate concentrations (0.5 mmol/l) of GO and MGO inhibit the EGFR kinase activity but do not alter the level of the EGFR protein.

However, it may be noted that high concentrations of GO induced the formation of anti-EGFR immunoreactive high-molecular-weight bands (arrow of Fig. 1*D*), suggesting the formation of high-molecular-weight complexes containing cross-linked and/or aggregated EGFR.

GO and MGO inhibit PTPase activity. Theoretically, the reduced EGF-induced EGFR autophosphorylation elicited by GO or MGO may result either from a defect of phosphorylation or an excess of dephosphorylation. Since recently reported data show that GO and MGO upregulated the activity of the protein phosphatase MKP-1 (26), we examined whether the aldehydes may also activate PTPases and the rate of EGFR dephosphorylation. Determination of overall cellular PTPase activity showed that under the conditions used, pretreatment of cells with GO (and MGO, data not shown) induced a severe inhibition of PTPase activity under both basal and EGF-stimulated conditions (Fig. 3*A*). Conversely, pulse-chase experiments, in which cells were preincubated or not with MGO, then stimulated with EGF, and then chased for variable time, showed that in MGO-treated cells, EGFR phosphorylation

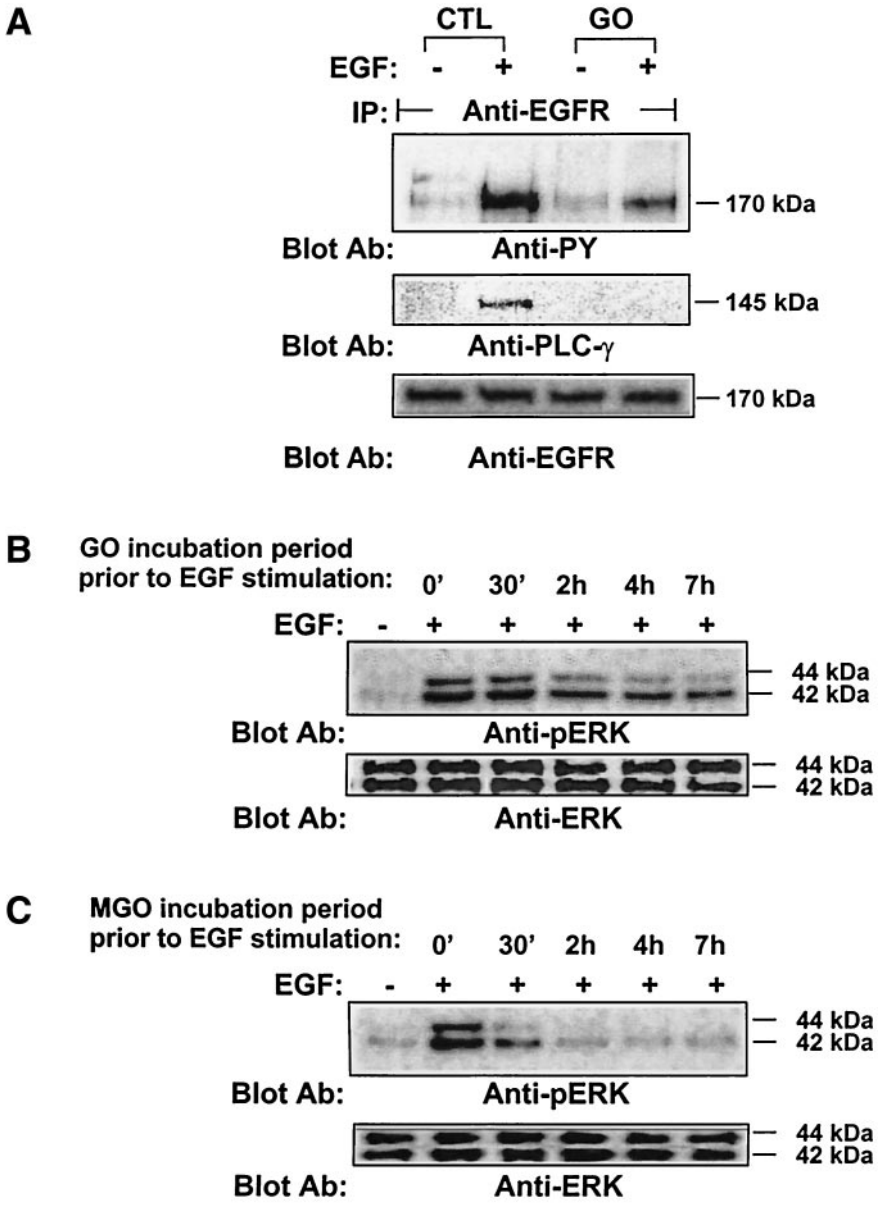

FIG. 2. GO and MGO alter EGFR downstream signaling. *A*: Inhibition of EGF-induced recruitment of PLCγ1 on EGFR. ECV304 cells were incubated with GO (5 mmol/l for 6 h) and then stimulated by EGF (10 nmol/l for 15 min). The cells were lysed in solubilization buffer (which allows coimmunoprecipitation of SH2-proteins bound to EGFR) and (1.5 mg cell protein) immunoprecipitated with anti-EGFR antibody overnight at 4°C. Anti-EGFR immunoprecipitates were recovered on protein G-Sepharose (1 h at 4°C), washed, eluted, and analyzed by SDS-PAGE. The spots were revealed by immunoblotting with anti-phosphotyrosine, anti-EGFR, and anti-PLCγ1. Data shown is representative of three experiments. *B* and *C*: Time course of inhibition of ERK activation subsequent to EGF stimulation. ECV304 cells, preincubated at variable periods of time with 5 mmol/l GO (*B*) or MGO (*C*), were stimulated with EGF (10 nmol/l for 15 min), and ERK activation was investigated by Western blot using antibodies against activated ERK (anti-phosphorylated ERK1/ERK2 [anti-pERK]) and total ERK (anti-ERK1/ERK2 [anti-ERK]). These data are representative of three separate experiments.

was lower but was maintained for a longer time (Fig. 3*B*). This MGO-induced delay of EGFR dephosphorylation was consistent with the inhibition of PTPase activity.

Therefore, inhibition by GO/MGO of the EGF-induced EGFR autophosphorylation does not result from an accelerated dephosphorylation due to PTPase activation. This led us to investigate whether GO or MGO directly inhibit EGFR kinase.

GO and MGO directly inhibit EGFR phosphorylation and tyrosine kinase activation. To investigate whether GO and MGO act directly on EGFR, we evaluated in vitro the EGFR kinase activity of EGFR immunopurified from ECV304 cells preincubated with GO and MGO. As shown in Fig. 4*A* and *B*, preincubation with MGO and GO inhibited the tyrosine kinase activity of EGFR (evaluated by EGFR autophosphorylation and phosphorylation of poly-Glu-Tyr). MGO was more active than GO.

All in vitro experiments gave similar results. In vitro preincubation of EGFR-rich membrane fractions from A431 cells with GO inhibited the in vitro EGF-induced EGFR autophosphorylation (Fig. 4*C*). Furthermore, EGFR tyrosine kinase activity was also decreased by GO and MGO in this system (data not shown).

All these data suggest that both GO and MGO (the latter

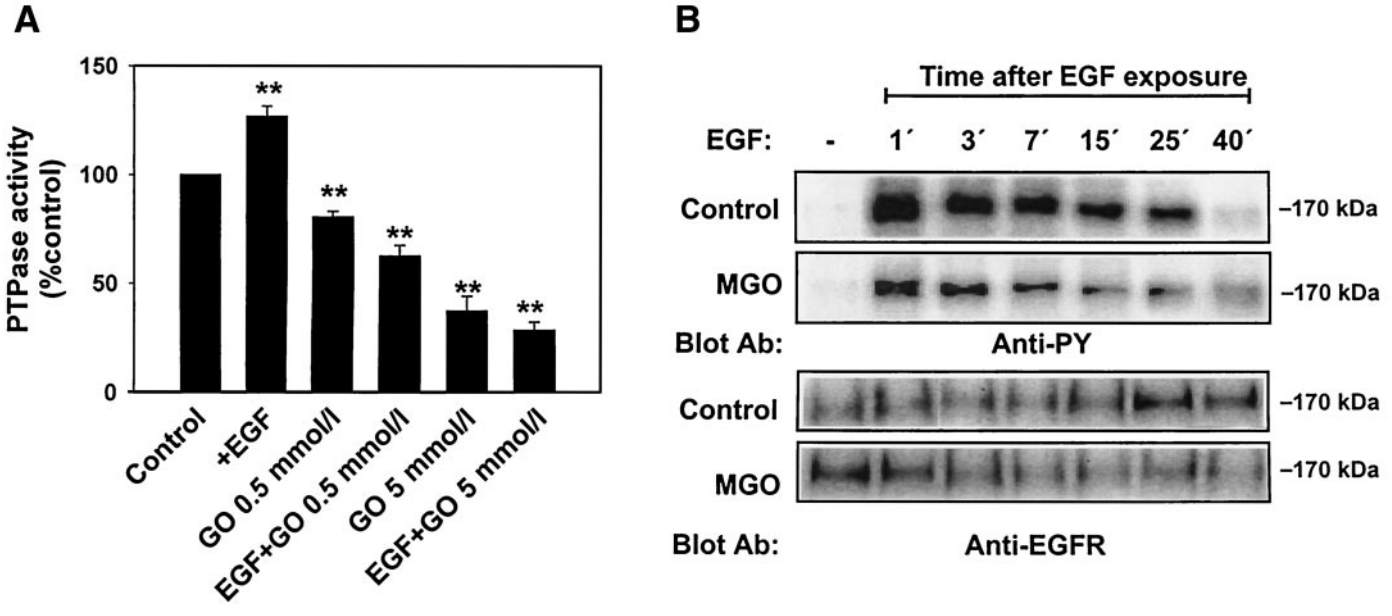

FIG. 3. Altered PTPase activity and EGFR dephosphorylation in cells incubated with GO and MGO. *A*: PTPase activity was evaluated in ECV304 cells preincubated (or not) with GO (0.5 and 5 mmol/l) for 5 h. PTPase activities were measured as described in RESEARCH DESIGN AND METHODS by dephosphorylation of [^{33}P]poly-Glu-Tyr. Data are expressed as percent of PTPase activation versus the basal control. Results are the means ± SD from four separate experiments. **$P < 0.001$ vs. unstimulated cells (control) (ANOVA). *B*: Inhibition of EGFR dephosphorylation by MGO. EGF (10 nmol/l) was added to ECV304 cells on ice. After binding on ice for 15 min, the cells were washed, chased at 37°C in EGF-free medium for the time periods indicated, and lysed in solubilizing buffer. After equilibration of protein concentration, cell lysates were analyzed by Western blot with an anti-phosphotyrosine antibody and developed by ECL as described in the text. These data are representative of three separate experiments.

being more efficient) directly inhibit EGFR autophosphorylation and tyrosine kinase activity. The in vitro inhibition of EGFR and the formation of high-molecular-weight aggregates of EGFR (detected by anti-EGFR antibodies) (Fig. 4*C*, lower panel) led us to hypothesize that GO and MGO may react with EGFR.

Derivatization of EGFR and cell proteins by GO and MGO: prevention by aminoguanidine

Formation of GO- and MGO-EGFR adducts. GO and MGO are highly reactive compounds, capable of reacting with free amino groups of cell proteins and forming AGEs (8). To evaluate whether GO and MGO derivatized basic amino acids of EGFR, we titrated the free reactive amino group content using the amine-reactive probe [^{3}H]SP (23). Both GO and MGO induced a significant reduction in the free amino group content of EGFR (Fig. 5*A*). These data suggest that preincubation of cells with GO or MGO induced the formation of GO- and MGO-EGFR adducts. CML is an AGE product that arises from the reaction of GO with lysine. The presence of the GO-derived AGE was

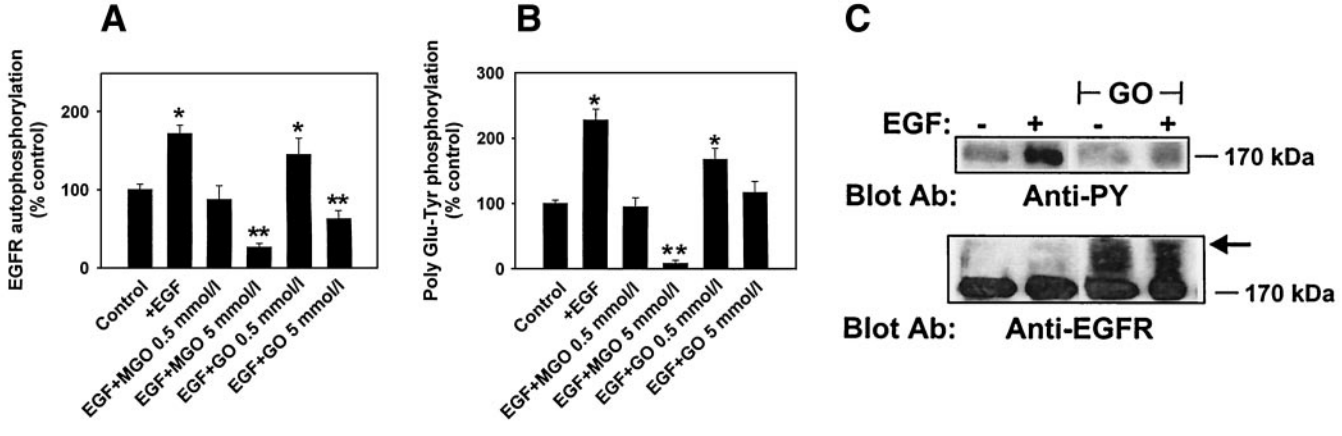

FIG. 4. In vitro inhibition of EGFR activation induced by GO and MGO. *A*: EGFR autophosphorylation was determined on EGFR immunopurified from unstimulated ECV304 (previously incubated in situ without or with 0.5 and 5 mmol/l GO and MGO for 5 h) and incubated in vitro without (control) or with 1 nmol/l EGF for 15 min. The assay was performed in phosphorylation buffer containing 5 μCi/assay of [γ-^{33}P]ATP, as described in RESEARCH DESIGN AND METHODS. After spotting on phosphocellulose membranes, the radioactivity was counted. Data are expressed as percent of the unstimulated control. *B*: EGFR tyrosine kinase activity was evaluated using the same protocol but in the presence of the poly-Glu-Tyr substrate. Results are the means ± SD from four separate experiments. *$P < 0.02$, **$P < 0.001$ vs. unstimulated cells (control) (ANOVA). *C*: Western blot experiments of EGFR in membranes prepared from A-431 cells and preincubated in phosphorylation buffer with or without 5 mmol/l GO (30 min), then stimulated with 1 nmol/l EGF (15 min), and then blotted with anti-phosphotyrosine and anti-EGFR antibodies. The arrow indicates the presence of anti-EGFR reactive high-molecular-weight aggregates. Data are representative of three separate experiments.

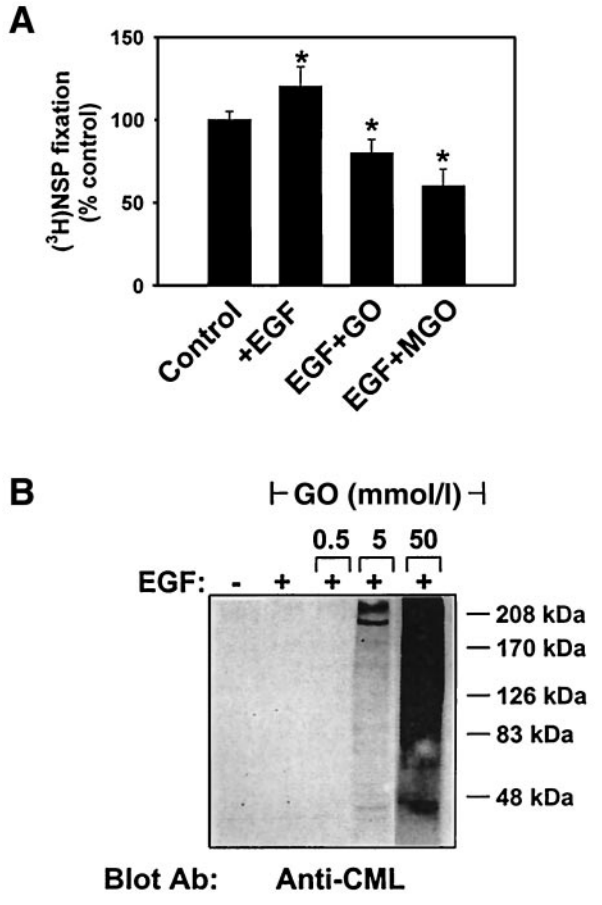

FIG. 5. Inhibitory effect of GO and MGO is related to AGE formation in EGFR. *A*: Derivatization of EGFR free amino groups by GO and MGO. ECV304 cells preincubated with GO or MGO (5 mmol/l for 5 h) were treated without (control) or with EGF (1 nmol/l for 15 min), and EGFR was immunoprecipitated as described above. Free amino groups of EGFR were labeled with [^{3}H]SP, as indicated in RESEARCH DESIGN AND METHODS. EGFR was resolved by SDS-PAGE, and the radioactivity of the 170-kDa bands was counted. Results are the means ± SD from three separate experiments. *P < 0.02 vs. unstimulated cells (control) (ANOVA). *B*: Detection of CML adducts by anti-CML antibodies in proteins from ECV304 cells preincubated with GO for 5 h at the indicated concentrations. Data are representative of four separate experiments.

confirmed by Western blotting of proteins from cells preincubated with GO (Fig. 5*B*) by anti-CML antibody.

Aminoguanidine prevents the formation of GO- and MGO-EGFR and protein adducts. Since aminoguanidine is able to prevent protein derivatization by dicarbonyl compounds (27,28), we investigated whether aminoguanidine can protect EGFR function against GO and MGO. As shown in Fig. 6, preincubation of cells with aminoguanidine before treatment by GO and MGO resulted in the protection of EGF-induced activation of EGFR and ERK1/2. This protection was almost complete against 5 mmol/l GO and partial against 5 mmol/l MGO.

Taken together, these data strongly suggest that the inhibitory action of GO and MGO on EGFR signaling was related to EGFR modification by these two AGE precursors and that this inhibitory effect can be prevented by aminoguanidine.

DISCUSSION

The reported data show, for the first time, that activation of the EGFR signaling pathway is inhibited by the dicarbonyl compounds GO and MGO in a time- and dose-dependent manner. The novelty of our results lies in the demonstration that EGFR is a target of GO and MGO, that GO and MGO induce the formation of GO/MGO-EGFR adducts and abrogate EGF-induced EGFR autophosphorylation and the activation of two of the major EGFR downstream signaling pathways, namely ERK and PLCγ, and that the formation of GO/MGO-EGFR (and GO/MGO–protein adducts) and subsequent inhibition of EGFR signaling can be prevented by aminoguanidine.

This aldehyde-induced inhibition is a novel mechanism of negative regulation of EGFR signaling. Because receptor protein tyrosine kinase signaling systems allow the cell to integrate a multitude of signals from its environment, the impairment of these basic mechanisms by reactive aldehydes could have profound effects on cellular physiology, including motility and cell fate determination such as differentiation, proliferation, and apoptosis. This concept agrees with the recently described role of MGO as a putative signal molecule in cell death (29).

Our results establish a clear relationship between molecular modifications (i.e., GO/MGO-EGFR adduct formation) and functional alterations (i.e., impairment of the EGFR signaling pathway). Under the conditions used, GO and MGO are able to derivatize EGFR, as assessed by a loss in [^{3}H]SP-reactive amino groups content of EGFR and by AGE-protein formation, as visualized by anti-CML antibody. This is consistent with the known reactivity of GO/MGO with amino groups of proteins (5). Reaction of GO/MGO with EGFR is also evidenced by the formation of high-molecular-weight aggregates reacting with anti-EGFR antibody. This is in agreement with the well-known association between AGE adducts and cross-linking of proteins (30).

Derivatization of EGFR by dicarbonyls and inhibition of EGFR kinase activity are concomitant events that are probably causally related, as suggested by in vitro experiments on purified EGFR and by the protective effect of aminoguanidine, a known dicarbonyl scavenger (27,28). Dicarbonyls may induce nonenzymatic modifications of various amino acids (Cys, Lys, or Arg) that are generally present in the putative active site of the receptor tyrosine

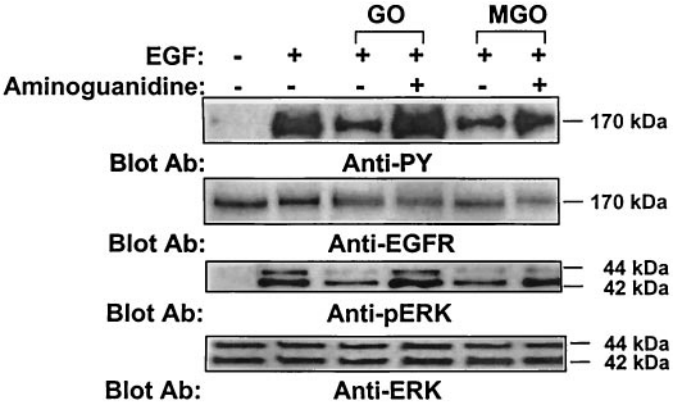

FIG. 6. Aminoguanidine prevents GO- and MGO-induced inhibition of EGF signaling in ECV304 cells. Cells were preincubated with or without aminoguanidine (5 mmol/l) for 8 h, exposed to GO or MGO (5 mmol/l for 5 h, as indicated), and stimulated with EGF. Analysis of EGFR phosphorylation and ERK phosphorylation was performed as described in RESEARCH DESIGN AND METHODS. Data are representative of four separate experiments.

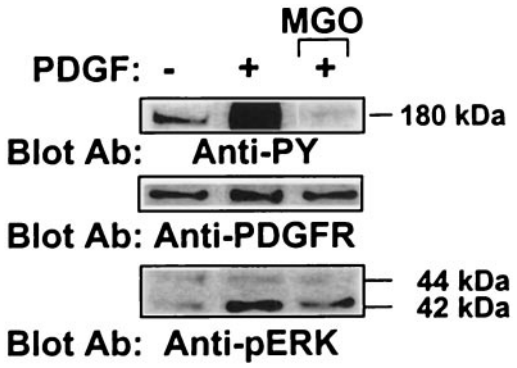

FIG. 7. MGO inhibits PDGFR autophosphorylation. Serum-starved rabbit smooth muscle cells (American *Type Culture* Collection) were preincubated with 3 mmol/l MGO for 5 h (as indicated) and stimulated with 1 nmol/l PDGF for 20 min. Thereafter, cells were lysed in solubilizing buffer, and equilibrated proteins were separated by SDS-PAGE (7.5%), as described in the legend to Fig. 1. Blots were probed with anti-phosphotyrosine, anti-PDGFR, and anti-phosphorylated ERK (anti-pERK) antibodies and developed by ECL as described. Data are representative of three separate experiments.

kinase family (31). Some other hypothetical mechanisms potentially involved in dicarbonyl-induced EGFR inhibition have been excluded: *1*) GO and MGO were not cytotoxic to cultured cells under the conditions used in the experiment; *2*) the loss of EGFR activity was not associated with a loss of EGFR protein but rather resulted from a reduced specific activity of the EGFR kinase; *3*) the GO/MGO–induced inhibition of EGFR phosphorylation does not result from activation of PTPases (involved in EGFR dephosphorylation) because, under our experimental conditions, GO/MGO strongly inhibited PTPases, in agreement with a previous report (32); and *4*) GO/MGO-induced EGFR inhibition is not prevented by antioxidants such as α-tocopherol and *N*-acetyl-cysteine (data not shown).

All these data strongly support the hypothesis that derivatization of EGFR by GO/MGO induces inhibition of EGFR kinase, thereby inhibiting EGFR autophosphorylation and downstream signaling pathway.

The broad capacity of dicarbonyls to induce protein derivatization suggests that GO/MGO may alter the function of other signaling proteins. This hypothesis was confirmed by the ability of GO/MGO to inhibit the activity of another tyrosine kinase receptor, the PDGFR in smooth muscle cells (Fig. 7). In the same way, the data on GO/MGO–induced dephosphorylation of ERKs (which are inhibited more rapidly than EGFR) suggest that other signaling proteins are targets for dicarbonyls. In this case, GO/MGO have been shown to activate MKP-1, a phosphatase involved in ERK dephosphorylation (26).

The reported GO/MGO–induced inhibition of EGFR and PDGFR signaling pathways observed in cultured cells is consistent with the growth factor resistance (including EGF) induced by high glucose concentrations in cultured fibroblasts (33).

The concentrations of GO/MGO used here are similar to those generally used to evaluate the biological effects of these dicarbonyls (10,11,26,34) and are consistent with those (high micromolar range) found in living systems (35). Moreover, these concentrations are close to those used in vitro to generate AGE content in a physiological range (8,36).

The GO/MGO–induced defects in cell signaling may be relevant to various steps of the pathophysiology of aging and diseases, with increased levels of dicarbonyls or AGEs, such as diabetes, uremia, and atherosclerosis (6,37). In diabetes, glycation of proteins is associated with increased generation of GO and MGO (3,38) and altered EGFR signaling (14–17). Concurrent to the AGE/RAGE–induced cell activation (39), this GO/MGO–induced impairment in response of growth factors and the subsequent trophic deprivation may be implicated in the pathophysiology of chronic diabetic complications, such as microvascular disease (including neuropathy, nephropathy, and retinopathy) and macrovascular disease (and associated accelerated atherosclerosis), impaired wound healing (33,40), and placenta dysfunction (16).

Finally, the protective effect of the dicarbonyl scavenger aminoguanidine, which prevents GO/MGO–induced EGFR signaling impairment, is also consistent with the effectiveness of aminoguanidine to slow the progression of a wide range of diabetic complications (41,42).

In conclusion, this study of the dicarbonyl-induced defect of growth factor response adds a new potential mechanism to the complex pathophysiology of chronic diabetic complications and other diseases associated with increased production of reactive aldehydes.

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