Original Article

Methylglyoxal Impairs the Insulin Signaling Pathways Independently of the Formation of Intracellular Reactive Oxygen Species

Audrey Riboulet-Chavey, Anne Pierron, Isabelle Durand, Joseph Murdaca, Jean Giudicelli, and Emmanuel Van Obberghen

Nonenzymatic glycation is increased in diabetes and leads to elevated levels of advanced glycation end products (AGEs), which link hyperglycemia to the induction of insulin resistance. In hyperglycemic conditions, intracellularly formed α-ketoaldehydes, such as methylglyoxal, are an essential source of intracellular AGEs, and the abnormal accumulation of methylglyoxal is related to the development of diabetes complications in various tissues and organs. We have previously shown in skeletal muscle that AGEs induce insulin resistance at the level of metabolic responses. Therefore, it was important to extend our work to intermediates of the biosynthetic pathway leading to AGEs. Hence, we asked the question whether the reactive α-ketoaldehyde methylglyoxal has deleterious effects on insulin action similar to AGEs. We analyzed the impact of methylglyoxal on insulin-induced signaling in L6 muscle cells. We demonstrate that a short exposure to methylglyoxal induces an inhibition of insulin-stimulated phosphorylation of protein kinase B and extracellular-regulated kinase 1/2, without affecting insulin receptor tyrosine phosphorylation. Importantly, these deleterious effects of methylglyoxal are independent of reactive oxygen species produced by methylglyoxal but appear to be the direct consequence of an impairment of insulin-induced insulin receptor substrate-1 tyrosine phosphorylation subsequent to the binding of methylglyoxal to these proteins. Our data suggest that an increase in intracellular methylglyoxal content hampers a key molecule, thereby leading to inhibition of insulin-induced signaling. By such a mechanism, methylglyoxal may not only induce the debilitating complications of diabetes but may also contribute to the pathophysiology of diabetes in general. *Diabetes* 55:1289–1299, 2006

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AGE, advanced glycation end product; CM-DCF, chloro-methyl-2′7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK, extracellular-regulated kinase; [^{3}H]SP, [^{3}H]succinimidyl propionate; IRS, insulin receptor substrate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PDTC, pyrrolidonedithiocarbamate; PI3K, phosphatidylinositol-3 kinase; p85, p85α regulatory subunit of PI3K; PKB, protein kinase B; PKC, protein kinase C; ROS, reactive oxygen species.

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Insulin plays a major role in regulating pathways leading to energy storage and to cellular proliferation and differentiation. Following insulin binding to its receptor, the receptor tyrosine kinase is activated, leading to the phosphorylation of several intracellular protein substrates. These rapid events generate multiple signaling cascades that eventuate in the final cellular responses to insulin (1,2). Insulin-activated signaling modules include the Ras/mitogen-activated protein kinase, the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB) and the protein kinase C (PKC) pathways (3,4). Resistance to insulin action is a common abnormality present in human disease situations such as diabetes and obesity. Insulin resistance associated with diabetes is thought to be genetically determined, but its occurrence is also clearly affected by environmental factors and processes secondary to diabetes itself (5). These acquired and secondary factors further impair insulin action in the diabetic patient. Thus, chronic hyperglycemia leading to insulin resistance is the primary etiological factor in the pathogenesis of diabetic microvascular disease and is implicated in macrovascular complications (6,7). One possible mechanism of hyperglycemia-induced tissue damage in diabetes is the formation of advanced glycation end products (AGEs). Chronic hyperglycemia augments nonenzymatic reactivity between reducing glucose and free amino groups of proteins, leading to the irreversible formation of AGEs (7,8). Persistent intracellular hyperglycemia appears to constitute the initiating event in the formation of both intracellular and extracellular AGEs (9), and a substantial amount of data indicate that glucose toxicity may be the consequence of increased formation of reactive α-ketoaldehydes such as 3-deoxyglucosone, glyoxal, and methylglyoxal (10). Methylglyoxal, physiologically present in all biological systems, is formed by the enzymatic and nonenzymatic elimination of a phosphate from triose-phosphate glycolytic intermediates and by oxidation of aminoacetone formed in the catabolism of threonine (11). Under physiological conditions, methylglyoxal is converted and detoxified by the glyoxalase system, which is present in the cytosol of all mammalian cells (12). In insulin-resistant states, like obesity and type 2 diabetes, altered glucose metabolism may lead to increased formation of methylglyoxal and other α-ketoaldehydes. Indeed, the concentration of methylglyoxal in the plasma of diabetic patients is increased proportionally with the degree of hyperglycemia (13). Further, in cultured endothelial

cells, under hyperglycemic conditions, it has been demonstrated that methylglyoxal accumulates rapidly and constitutes the most important precursor in the nonenzymatic glycation of proteins to yield irreversible AGEs (14). These modifications affect the properties of the proteins and thus alter intracellular events. Although methylglyoxal and its AGE counterparts have been linked to vascular complications of diabetes, it is currently unclear whether exposure to methylglyoxal induces changes in insulin action and at which step in the insulin-signaling pathways such putative modifications may occur. The aim of our present work is to identify which intracellular proteins, implicated in insulin signaling, are modified by an increase in intracellular methylglyoxal and to determine the consequences in the biological responses induced by insulin. To investigate such deleterious action of methylglyoxal, we analyzed the effects of methylglyoxal on insulin signaling in L6 muscle cells. We show that an increase in the intracellular methylglyoxal concentration induces an insulin-resistant state in L6 muscle cells by interfering with key insulin-signaling molecules.

RESEARCH DESIGN AND METHODS

Chemicals. Media and sera for cell culture were from Life Technologies (Grand Island, NY). Chloro-methyl-2′7′-dichlorofluorescein diacetate (CM-DCF) was from Molecular Probes (Eugene, OR). Phospho-PKB and phospho-extracellular-regulated kinase (ERK) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Insulin receptor substrate (IRS)-1 and p85α regulatory subunit of PI3K (p85) antibodies were from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antiserum to IRS-1, used for immunoprecipitation experiments, was produced in our laboratory. All other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Electrophoresis and Western blot reagents were from BioRad (Richmond, VA), and protein A Sepharose was from Pierce (Rockford, IL). [2-^{14}C]methylglyoxal (55 mCi/mmol) was manufactured by American Radiolabeled Chemicals (St. Louis, MO). [^{3}H]-2-deoxy-D-glucose, [H^{3}]succinimidyl propionate ([H^{3}]SP) were from Amersham Pharmacia Biotech (Arlington Heights, IL). Methylglyoxal, aminoguanidine, 2-deoxy-D-glucose, and all other reagents were from Sigma (St. Louis, MO).

Cell culture. Rat L6 myoblasts were obtained from the American Type Culture Collection (CRL 1458; Rockville, MD). The L6 muscle cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 100 units/ml penicillin, 100 μg/ml streptomycin, 10% (vol/vol) heat-inactivated FCS, and 2 mmol/l glutamine. Cultures were maintained at 37°C in a humidified atmosphere containing 5% (vol/vol) CO_2. Cells were starved for 4 h in serum-free DMEM supplemented with 0.2% (wt/vol) BSA before addition of methylglyoxal at times and concentrations indicated below.

Assessment of cell viability and necroses. To test whether methylglyoxal has toxic effects in L6 cells, we performed a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay as described (15). Briefly, L6 cells were seeded in DMEM containing 10% (vol/vol) FCS at a density of 10^4 cells per well into 24-well culture plates and were grown for 24 h. Medium was then replaced, and the cells were incubated in the absence or presence of inhibitors for 30 min before being treated or not (control) with methylglyoxal at various concentrations. After incubation, viability of the cells was assessed using MTT. The activity of lactate dehydrogenase present in the incubation media at the beginning and end of all methylglyoxal incubations was evaluated as an index of necrosis (16). In addition, possible methylglyoxal-induced apoptosis was evaluated by caspase activity determination as described in (17).

2-deoxy-D-glucose uptake. Glucose uptake measurement was performed in L6 cells as previously described (18). Briefly, L6 cells were cultured in 12-well plates and, after a 4-h starvation period, pretreated or not with methylglyoxal as indicated. The cells were washed with starvation medium and then exposed or not to 100 nmol/l insulin for 20 min. Glucose uptake was measured by incubating the cells for 5 min in HEPES buffer (140 mmol/l NaCl, 20 mmol/l HEPES, 2.5 mmol/l MgSO4, 1 mmol/l CaCl, and 2.5 mmol/l KCl, pH 7.4) containing 10 μmol/l [^{3}H]-2-deoxy-D-glucose (1 μCi/assay). The reaction was stopped by addition of 10 μmol/l cytochalasin B, and cells were then washed three times with ice-cold isotonic saline solution before lysis in 0.1 mol/l NaOH. After neutralization, radioactivity was measured by liquid scintillation and corrected for total cellular protein content.

Immunoblot analysis. After treatment with methylglyoxal in the absence or presence of inhibitors, the cells were washed with starvation medium and then exposed or not to 100 nmol/l insulin for 10 min in the absence or presence of inhibitors. Cells were scraped and solubilized for 20 min at 4°C with lysis buffer (50 mmol/l HEPES, 150 mmol/l NaCl, 10 mmol/l EDTA, 10 mmol/l $Na_4P_2O_7$, 2 mmol/l sodium orthovanadate, 50 mmol/l NaF, 1% [vol/vol] Triton X-100, pH 7.4, containing 1 mmol/l 4-[2-aminoethyl]-benzenesulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Whole-cell lysates were cleared by centrifugation at 12,000*g* for 15 min at 4°C and were either used directly or subjected to immunoprecipitation with antibodies to IRS-1. Proteins from whole-cell lysates or immunoprecipitates were separated on 7.5% SDS-PAGE, transferred to nitrocellulose, and blotted with various antibodies (as specified under RESULTS). After incubation with secondary antibody, blots were processed for enhanced chemiluminescence (Amersham Pharmacia Biotech), and bands were quantitated by densitometry using the ImageQuant 5.1 program.

Assay for PI3K activity. PI3K assays were performed as previously reported (19). Briefly, L6 myoblasts were deprived of serum for 4 h in the presence or absence of methylglyoxal and then exposed to 100 nmol/l insulin for 10 min. After insulin stimulation, the cells were solubilized for 20 min at 4°C in TAT buffer. After lysate clarification by centrifugation at 12,000*g* for 15 min at 4°C, aliquots of the lysates were subjected to immunoprecipitation with anti–IRS-1 antibodies coupled to protein A-Sepharose for 2 h at 4°C. PI3K activity was determined in immunoprecipitates.

Cell fractionation and membrane isolation. L6 cells from 150-mm diameter Petri plates (Falcon) were treated and plasma membranes prepared as previously described (20). Briefly, after homogenization with a Dounce homogenizer, a 1,000*g* centrifugation to remove unbroken cells and nuclei was performed. The microsomal and cytosolic fractions were separated by centrifugation of the postnuclear supernatant at 27,000*g* for 30 min at 4°C.

Measurement of reactive oxygen species production. Intracellular production of reactive oxygen species (ROS) was measured by CM-DCF fluorescence as described (21). Cells were treated with methylglyoxal for various lengths of time at the concentrations indicated, in the absence or presence of 200 μmol/l of the antioxidant pyrrolidonedithiocarbamate (PDTC), and then exposed to 50 μmol/l CM-DCF in PBS for 45 min. Cells were washed three times with PBS, swelled in H_2O, and sonicated. The fluorescence was determined at 488/525 nm, normalized on a protein basis, and expressed as a percentage of the control.

Derivatization of free amino groups on IRS-1. After preincubation of L6 cells with 5 mmol/l methylglyoxal, in absence or presence of inhibitors for 30 min, IRS proteins were immunoprecipitated, and the free amino group content was evaluated by use of amine-reactive probe 0.5 mol/l [^{3}H]SP in borate buffer, pH 8.5, as previously described (22). Immunoprecipitates were washed three times in borate buffer and boiled in 4 × Laemmli buffer. IRS proteins were resolved by SDS-PAGE, the higher part of polyacrylamide gel, containing IRS proteins, was recovered, and the radioactivity was counted by liquid scintillation.

Statistical analysis. Results are expressed as means ± SD, and statistical analysis was performed using the Student's *t* test with a statistical significance of at least $P < 0.05$.

RESULTS

Incorporation of [2-^{14}C]methylglyoxal into L6 cells. To determine the possible effects of an increase in intracellular methylglyoxal concentrations on insulin action, the incorporation of methylglyoxal into L6 cells was measured after addition of various concentrations of [2-^{14}C]methylglyoxal to the culture medium. The results obtained suggest that only 3.1 ± 0.39%, which corresponds to ~100 μmol/l of the 2.5 mmol/l of the methylglyoxal added to the conditioned medium, was incorporated into the cells and may eventually be involved in generation of intracellular effects in L6 cells (Fig. 1). Indeed, under the conditions used, no radioactivity was detected in the final wash buffer, indicating that the radioactivity observed associated with the cells is not the consequence of a contamination by free radioactivity. Similar observations have been reported concerning the incorporation of radiolabeled methylglyoxal into rat aortic smooth muscle cells (23). After cellular fractionation of the L6 cells, ~32.8 ± 2.5 and 63.5 ± 9.5% of radiolabeled methylglyoxal incorporated into the cells were found, respectively, in cytosolic

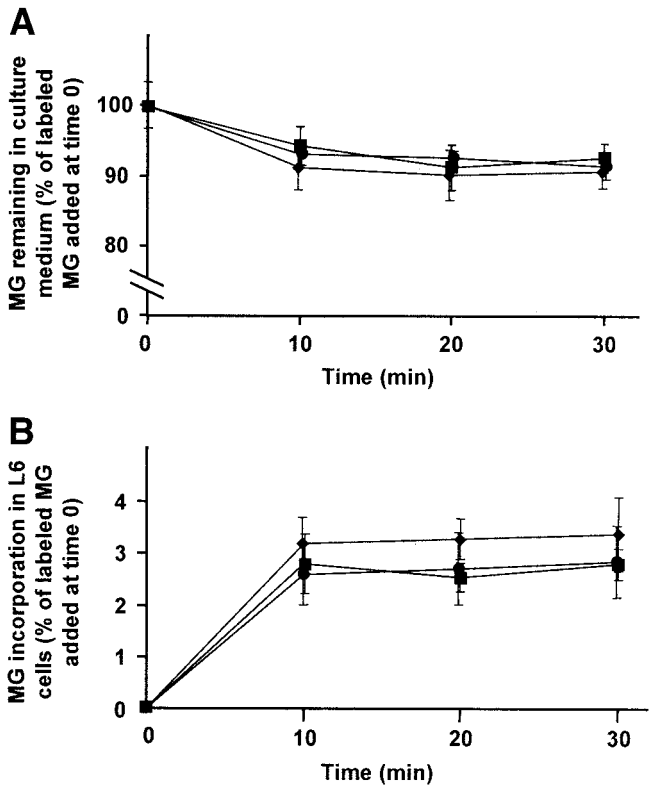

FIG. 1. Incorporation of [2-^{14}C]methylglyoxal into L6 cells. After incubation of L6 cells with 2.5 mmol/l [^{14}C]methylglyoxal in starvation medium, the media were collected at the indicated times and then were centrifuged to remove cell debris. The cells in the plates were washed three times with ice-cold PBS to remove the nonspecifically bound methylglyoxal (MG) and lysed in 0.1 mol/l NaOH. The radioactivity of the starvation media remaining (*A*) and incorporated into the cells (*B*) were then measured. For incorporation at time 0, the cells were lysed without treatment with the labeled methylglyoxal. Symbols and bars represent the mean ± SD of quadruplate determinations obtained in three independent experiments.

and membrane fractions (data not shown). Interestingly, pretreatment of the cells with aminoguanidine (5 mmol/l), which reacts preferentially with dicarbonyl AGE precursors such as methylglyoxal and prevents modification of proteins by this dicarbonyl compound (24), reduced the incorporation of radiolabeled methylglyoxal into the cells (1.4 ± 0.2%) (data not shown).

Effects of methylglyoxal treatment on viability of L6 cells. Methylglyoxal has been shown to be toxic to cultured cells through the induction of ROS-mediated apoptosis (25–27). However, this cytotoxicity is usually observed after a long exposure (after 4–24 h) of cells to methylglyoxal (26,27). To test whether the concentration of methylglyoxal and the exposure times used in this study have toxic effects on L6 cells, we measured the following three parameters summarized in Table 1: *1*) The percentage of necrotic cells based on the measurement of lactate dehydrogenase activity in the incubation media at the beginning and end of all methylglyoxal incubations. The necrotic cell population remained at a very low level and did not increase significantly when the cells were treated for 1 h with 2.5 mmol/l methylglyoxal. *2*) The methylglyoxal-induced apoptosis by the determination of the caspase activity. No significant increase in caspase activity was observed in the methylglyoxal-treated cells. *3*) The viability of cells after treatment with methylglyoxal assessed by the MTT assay. The results obtained demonstrate that the viability was found to be equal to that seen in non–methylglyoxal-treated cells. To summarize, within 1 h treatment and at concentrations up to 2.5 mmol/l, methylglyoxal does not appear to have detectable deleterious effects on the viability of L6 cells. Similar resistance to cytotoxic effects of methylglyoxal has been described for isolated rat hepatocytes (28) and the human immortalized endothelial ECV304 cell line (29).

TABLE 1
Effects of methylglyoxal on L6 cell viability

Methylglyoxal (1 h; mmol/l)	0.5	1.0	2.5
LDH activity* (% of control)	<3%	<3%	<3%
Caspase activity† (% of control)	99 ± 4	99 ± 3	96 ± 3
Cell viability‡ (% of control)	97 ± 1.3	97 ± 1.9	94 ± 3.1

Data are means ± SD. *Data are expressed as percent of the maximal level of lactate dehydrogenase (LDH) activity determined after total cell lysis. †Data are expressed as percent of the caspase specific activity in control untreated cells. ‡The cell viability was measured by a MTT assay. Each experiment were performed in triplicate (*†) or duplicate (‡) from three independent experiments.

Insulin signaling is inhibited in short-term methylglyoxal-exposed cells. Insulin-resistant states are associated with altered glucose metabolism in muscle cells (30). To determine whether methylglyoxal could induce such alterations in L6 cells, we studied the effects of preincubation with 0.5–2.5 mmol/l methylglyoxal on glucose transport. In cells not treated with methylglyoxal, insulin induced a 2.3-fold increase in 2-deoxy-D-glucose uptake. Methylglyoxal decreased insulin-stimulated glucose uptake in a dose-dependent manner without affecting basal glucose uptake (Fig. 2*A*). To study the time course of the methylglyoxal-induced inhibition of glucose transport, cells were preincubated with 2.5 mmol/l methylglyoxal for up to 30 min and, after insulin stimulation, glucose uptake was measured. As shown in Fig. 2*B*, in the presence of methylglyoxal, insulin-induced glucose transport was progressively inhibited, and maximal inhibition was seen after 30 min of incubation. Interestingly, this inhibition of insulin-induced glucose transport was prevented when the cells were pretreated with aminoguanidine, a nucleophilic agent which, via its two aminogroups (hydrazine and guanidine), acts as reactive scavenger of α-ketoaldehyde glycating agents such as methylglyoxal, glyoxal, or 3-deoxyglucosone. Methylglyoxal has been described to induce ROS production (25–27). To determine whether reactive oxygen intermediates are potentially involved in inhibition of insulin-stimulated glucose transport, the cells were pretreated with the oxidative stress inhibitor PDTC, which counteracts the oxygen free radical effects. As shown in Fig. 2*A*, PDTC addition did not block the deleterious effect of methylglyoxal on insulin-stimulated glucose transport.

To investigate the mechanisms underlying this inhibition of insulin-stimulated glucose transport, the early molecular events in insulin signaling were compared in absence and presence of methylglyoxal. To study the insulin receptor modifications, L6 cells were treated with 5 mmol/l methylglyoxal for 30 min and with 2.5 mmol/l methylglyoxal for various periods of time (up to 30 min) to analyze the intracellular protein modifications. Thereafter, the cells were exposed to insulin for 10 min. Cell lysates were prepared, insulin receptor and IRS-1 proteins were immunoprecipitated with the corresponding specific antibodies, and the tyrosine phosphorylation status analyzed

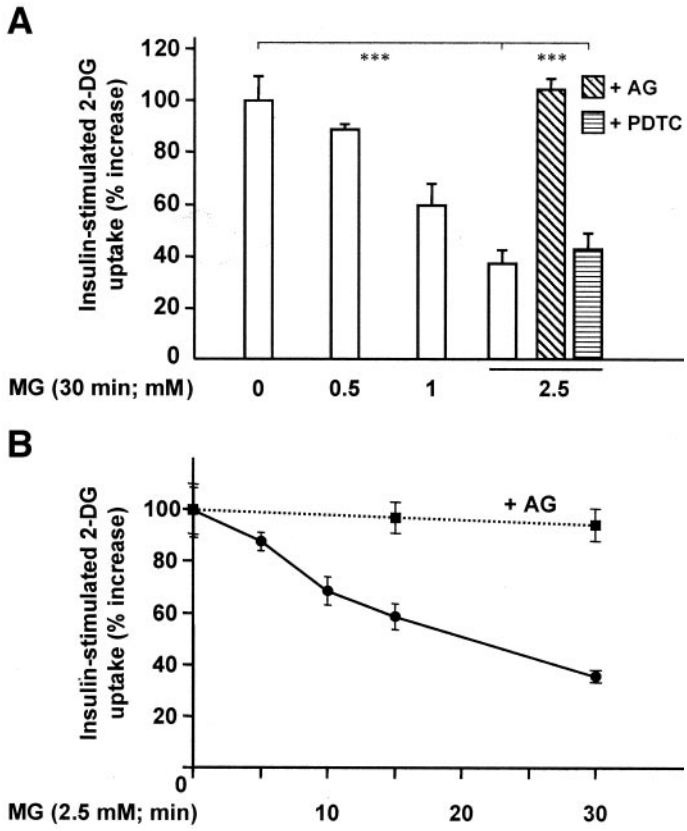

FIG. 2. Methylglyoxal (MG) action on 2-deoxy-D-glucose uptake in L6 cells. L6 cells were incubated without or with methylglyoxal at different concentrations (*A*) and for different periods of time (*B*) and then stimulated with 100 nmol/l insulin for 20 min. 2-deoxy-D-glucose (2-DG) uptake was determined as described in RESEARCH DESIGN AND METHODS. Symbols and bars represent the mean ± SD of triplicate determinations obtained in three independent experiments. Significant as compared with the insulin-stimulated glucose transport in absence of methylglyoxal (*$P < 0.001$).**

by Western blotting. Methylglyoxal treatment (up to 5 mmol/l) did not impair the ability of insulin to stimulate its receptor autophosphorylation (Fig. 3*A*). On the contrary, insulin-dependent IRS-1 tyrosine phosphorylation was severely reduced in cells preincubated with methylglyoxal. Indeed, methylglyoxal action on IRS-1 phosphorylation resulted in a progressive decrease in insulin-stimulated phosphorylation over time, with maximum inhibition (approximately threefold) reached within 30 min of methylglyoxal exposure. Quantification of the total IRS-1 protein levels revealed that the methylglyoxal-induced decrease in tyrosine phosphorylation of IRS-1 did not result from a reduction in this protein, as no significant differences in protein levels were observed in the methylglyoxal-treated group as compared with the untreated group. Further, this decreased IRS-1 tyrosine phosphorylation was also not due to an alteration of insulin receptor activation (Fig. 3*B* and *C*). A similar reduction in IRS-2 tyrosine phosphorylation was observed in the methylglyoxal-treated cells (data not shown).

Recent studies have reported that serine phosphorylation of IRS-1 is a possible mechanism involved in the decrease in insulin-induced IRS-1 tyrosine phosphorylation (31). To determine whether the serine phosphorylation of IRS-1 was increased after methylglyoxal-exposure, L6 cells were treated with or without 2.5 mmol/l methylglyoxal for 30 min before insulin stimulation, and IRS-1 was immunopurified and immunoblotted with an antibody to phosphoserine. As shown in Fig. 3*D*, in response to methylglyoxal, no difference in mobility of IRS-1, characteristic of an increase in its serine/threonine phosphorylation, was observed. Immunoblotting with an antibody to phosphoserine confirms the lack of serine phosphorylation on IRS-1 in the methylglyoxal-treated cells. Taken together, these results indicate that methylglyoxal does not increase the serine phosphorylation of IRS-1. Similar results were obtained with IRS-2 in the methylglyoxal-treated cells (data not shown). As PI3K is known to be involved in mediating insulin-stimulated glucose uptake in muscle cells, we determined the PI3K activity in IRS-1 immune complexes. As expected, insulin induced a 2.7-fold increase in IRS-1–associated PI3K activity in control cells. However, the stimulatory action of insulin on PI3K activity was virtually abolished when cells were pretreated for 30 min with methylglyoxal. Importantly, IRS-1–associated PI3K activity was not affected by exposure to methylglyoxal when the L6 cells were preincubated with aminoguanidine (Fig. 3*E*).

To determine whether the methylglyoxal-inhibitory effects on PI3K are a consequence of an alteration in the association or activation of the lipid kinase, the levels of the regulatory α p85 subunit of PI3K in IRS-1 immunoprecipitates were determined in cells treated or not with different concentrations of methylglyoxal for 30 min. In the absence of methylglyoxal treatment, insulin induced a marked increase in p85 levels coimmunoprecipitated with IRS-1 (~6.5-fold the unstimulated level), and this was associated with the insulin-stimulation of IRS-tyrosine phosphorylation. In the presence of methylglyoxal, the levels of insulin-stimulated IRS-1 tyrosine phosphorylation decreased in a dose-dependent manner. Importantly, when proteins immunoprecipitated with antibodies to IRS-1 were analyzed by Western blot with antibodies to p85, we found that in addition, methylglyoxal was reduced in a dose-dependent manner the PI3K–IRS-1 association. This decrease becomes pronounced at 0.5 mmol/l methylglyoxal (~4.5-fold the unstimulated level), while the maximum inhibition is reached at 2.5 mmol/l methylglyoxal leading to an almost complete inhibition (Fig. 4*A*–*C*). Importantly, the reduced phosphorylation of IRS-1 and IRS-1–p85 interaction were not associated with changes in expression levels of the proteins involved (Fig. 4*A* and *D*). Finally, note that this decrease in PI3K-IRS association explains the loss of the stimulatory action of PI3K activity in cells treated with 2.5 mmol/l methylglyoxal for 30 min (Fig. 3*E*).

To evaluate whether the inhibition of insulin-dependent IRS-1 tyrosine phosphorylation was associated with alterations in downstream signaling, the phosphorylation states of PKB, ERK1 (p44), and ERK2 (p42) were analyzed. Methylglyoxal treatment of L6 cells induced a dose-dependent inhibition of insulin-stimulated phosphorylation of PKB on Ser^{473} (Fig. 5*A*). The stimulatory action of insulin on phosphorylation of PKB on Ser^{473} and Thr^{308} was virtually completely inhibited after treatment of the cells with 0.5 mmol/l methylglyoxal for 30 min. A progressive decrease in insulin-stimulated phosphorylation of PKB over time was observed, resulting in significant inhibition after 10 min and maximum inhibition after 30 min incubation with 2.5 mmol/l methylglyoxal (Fig. 5*B*). Note that the inhibition of the phosphorylation of PKB on serine and threonine is prevented when aminoguanidine is added

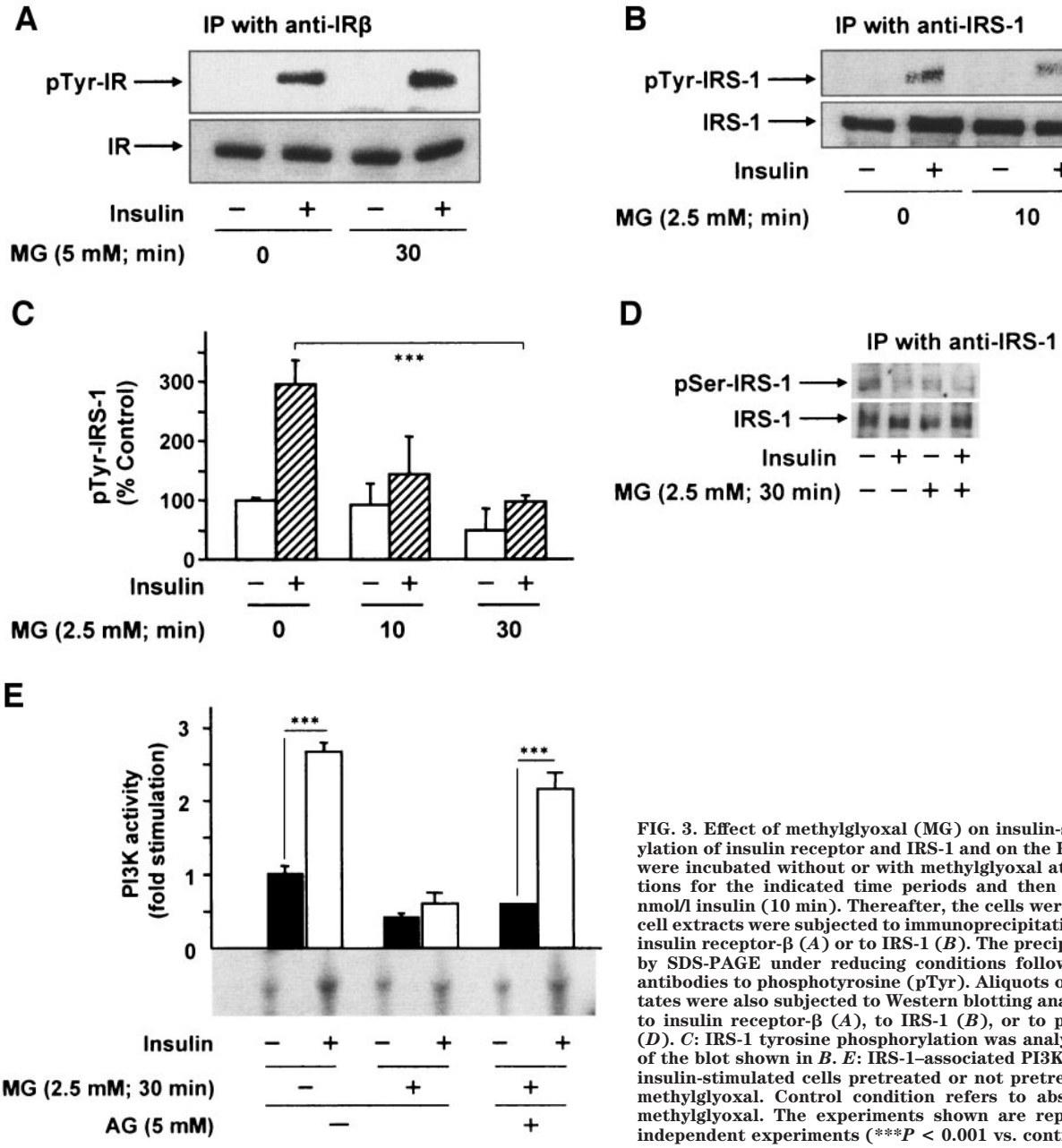

FIG. 3. Effect of methylglyoxal (MG) on insulin-stimulated phosphorylation of insulin receptor and IRS-1 and on the PI3K activity. L6 cells were incubated without or with methylglyoxal at different concentrations for the indicated time periods and then stimulated with 100 nmol/l insulin (10 min). Thereafter, the cells were solubilized, and the cell extracts were subjected to immunoprecipitation with antibodies to insulin receptor-β (*A*) or to IRS-1 (*B*). The precipitates were analyzed by SDS-PAGE under reducing conditions followed by blotting with antibodies to phosphotyrosine (pTyr). Aliquots of the immunoprecipitates were also subjected to Western blotting analysis with antibodies to insulin receptor-β (*A*), to IRS-1 (*B*), or to phosphoserine (pSer) (*D*). *C*: IRS-1 tyrosine phosphorylation was analyzed by densitometry of the blot shown in *B*. *E*: IRS-1–associated PI3K activity in basal and insulin-stimulated cells pretreated or not pretreated with 2.5 mmol/l methylglyoxal. Control condition refers to absence of insulin and methylglyoxal. The experiments shown are representative of three independent experiments (*$P < 0.001$ vs. control).**

before and during the incubations with methylglyoxal (Fig. 5*B*).

To determine whether methylglyoxal could induce alterations in the phosphorylation of ERK1/2, the effects of preincubation for 30 min with 0–2.5 mmol/l methylglyoxal on ERK1/2 phosphorylation were studied. The basal phosphorylation level of ERK1/2 was relatively high in the untreated control cells (Fig. 5*A* and *B*). After 30 min methylglyoxal treatment, a dose-dependent modification in ERK1/2 phosphorylation occurred, and levels of insulin-unstimulated phosphorylated ERK1/2 were significantly increased by treatment with 1 mmol/l or 2.5 mmol/l of methylglyoxal in L6 cells (Fig. 5*A*). To study the time course for methylglyoxal-induced changes in ERK1/2 phosphorylation, cells were preincubated with 2.5 mmol/l methylglyoxal for 10 or 30 min and then stimulated or not with insulin for 10 min. In unstimulated cells, the basal phosphorylation level of ERK1/2, which is not modified after 10 min incubation with methylglyoxal, was significantly increased by ~1.5-fold upon methylglyoxal incubation for 30 min (increase significant at the $P < 0.01$ level). This methylglyoxal-induced phosphorylation of ERK1/2 is prevented if the cells are pretreated with aminoguanidine (Fig. 5*B*). Data presented in Fig. 5*B* and *C* show that the insulin-stimulated phosphorylation of ERK1/2 was significantly increased (~1.26-fold the insulin-stimulated control) after 10 min of methylglyoxal treatment ($P < 0.05$) but decreased within 30 min of methylglyoxal exposure ($0.38 \pm 0.05\%$ of the insulin-stimulated control). Quantification of total ERK1/2 proteins indicated that the methylglyoxal-induced effects on phosphorylation of ERK1/2 did not result from a reduction in these proteins.

Recently, ERK1/2 have been implicated in the down-regulation of insulin signaling (32,33). To evaluate the role of ERK1/2 on the methylglyoxal-induced inhibition of the insulin action, a kinase inhibitor or aminoguanidine were

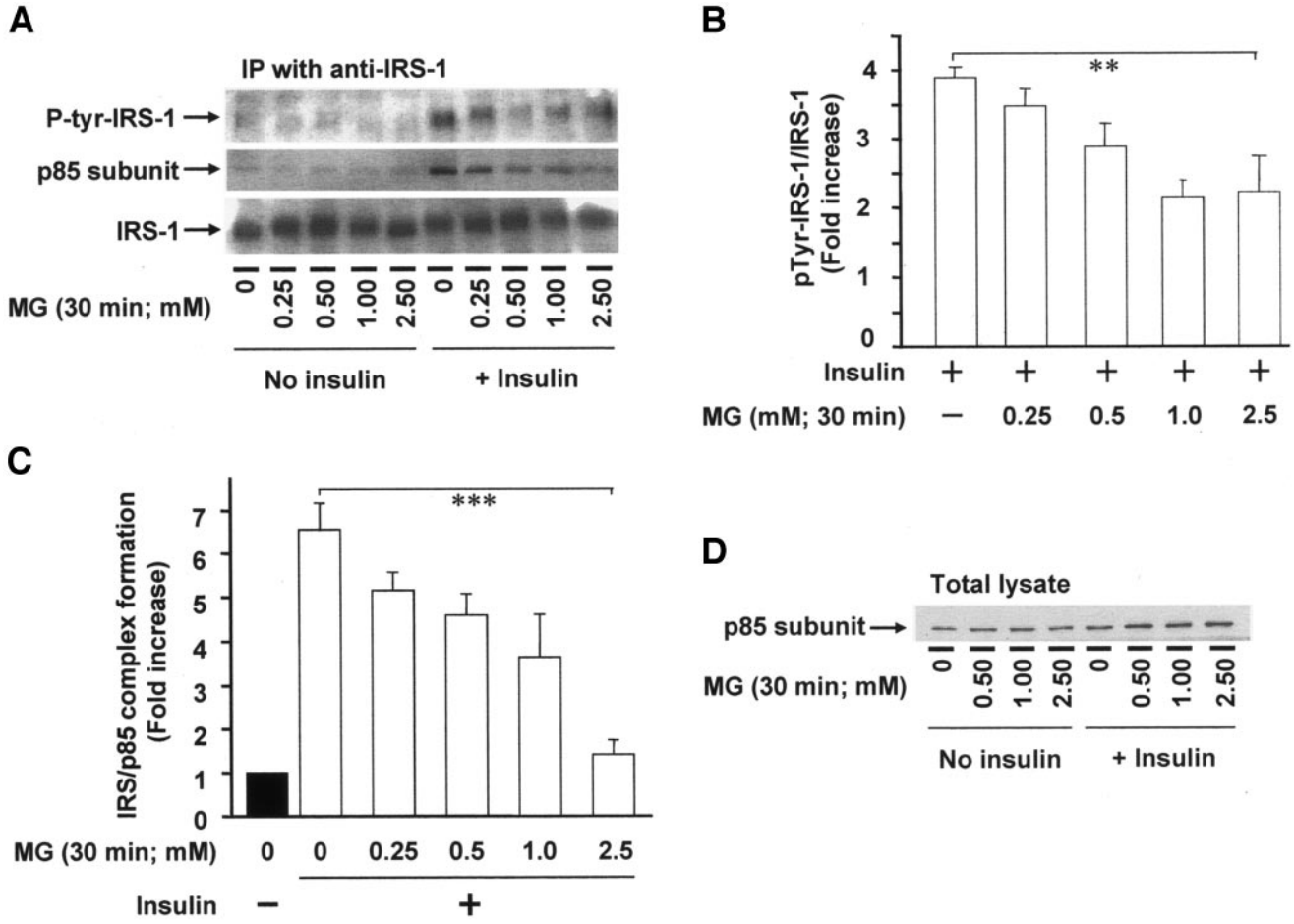

FIG. 4. Dose-dependent effects of methylglyoxal (MG) on insulin-stimulated phosphorylation of IRS-1 and IRS-1/p85 association in L6 cells. L6 cells were treated as described in Fig. 3. Thereafter, the cells were solubilized and the cell extracts were subjected to immunoprecipitation with antibodies to IRS-1 and then the precipitates analyzed by SDS-PAGE under reducing conditions followed by blotting with antibodies to phosphotyrosine (pTyr), p85, and IRS-1 (*A*). *B* and *C*: Results of densitometric analyses of three independent experiments. ■, unstimulated conditions; □ stimulated conditions (*P* < 0.01; ****P* < 0.01). Aliquots of lysates were also immunoblotted for p85 (*D*).**

added in the incubation medium before and during the incubation with 2.5 mmol/l methylglyoxal. Incubation of cells with PD98059, a selective inhibitor of MEK1, for 30 min before methylglyoxal treatment resulted in complete inhibition of ERK1/2 phosphorylation. The methylglyoxal effects on phosphorylation of ERK1/2 can be prevented when aminoguanidine is added before and during the incubation with methylglyoxal (Fig. 6*A* and *B*). Importantly, unlike PD98059 inhibitor, aminoguanidine prevents the methylglyoxal-induced inhibition of PKB phosphorylation (Fig. 6*C*). Taken together, these results demonstrate that the inhibition of PKB phosphorylation, observed after methylglyoxal addition, is independent of the ERK1/2 pathway.

Specificity of methylglyoxal effects on the insulin-induced signaling pathways. Methylglyoxal has been shown to be toxic to cultured cells through the induction of ROS production (25–27). In addition, previous studies in 3T3-L1 adipocytes and Fao rat hepatoma cells have demonstrated that oxidative stress could induce concomitantly metabolic insulin resistance and increase IRS-1 degradation (34). To investigate whether the alterations observed on the early molecular events in insulin signaling are the consequence of an increase in ROS production, intracellular ROS levels were measured before and after treatment of cells by methylglyoxal. As shown in Fig. 7*A*, treatment of L6 cells with methylglyoxal induces a time- and dose-dependent production of ROS, which was prevented when cells were treated with aminoguanidine, a blocker of carbonyl groups, or with the antioxidant PDTC.

In our attempt to characterize the implications of methylglyoxal-induced ROS production in the alterations of insulin signaling pathways, we performed experiments in the presence of the antioxidants *N*-acetylcysteine (20 μmol/l), PDTC (200 μmol/l), and diphenyleneiodonium (100 μmol/l), a flavoprotein inhibitor. In unstimulated cells, these antioxidant compounds, added 30 min before and together with 2.5 mmol/l methylglyoxal, partially prevented the methylglyoxal-increased ERK1/2 phosphorylation. Note that in presence of aminoguanidine, ERK1/2 were phosphorylated by methylglyoxal treatment to a much lesser extent (Fig. 7*B* and *C*). Taken together, these results suggest that methylglyoxal induces ERK1/2 phosphorylation through ROS generation. The reduction of methylglyoxal action in presence of aminoguanidine is likely to be due to scavenging of methylglyoxal by aminoguanidine. Importantly, the coincubation with these antioxidant compounds had no significant protective action on the methylglyoxal-induced inhibition of PKB phosphorylation (Fig. 7*D*). These results demonstrate that the inhibition of PKB phosphorylation, observed after methylglyoxal addition, is independent of methylglyoxal-induced ROS production.

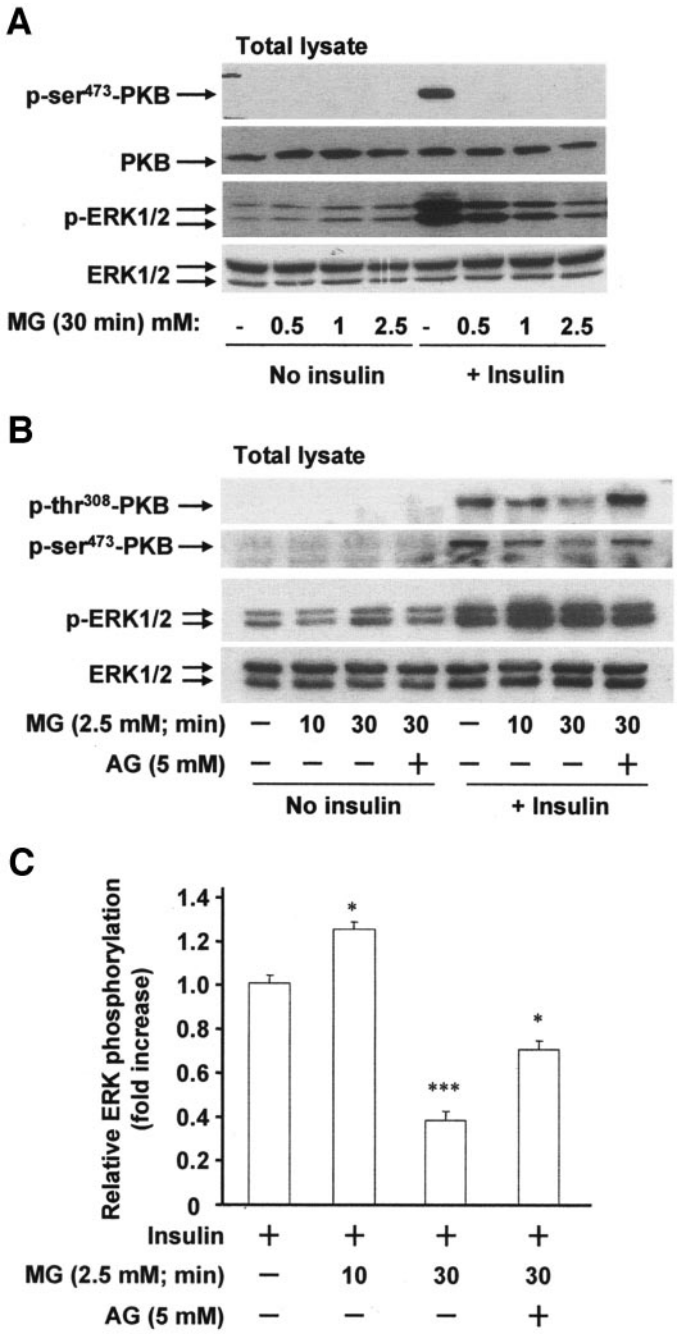

FIG. 5. Effects of methylglyoxal (MG) on insulin-stimulated phosphorylation of PKB and ERK1/2 in L6 cells. L6 cells were treated with different methylglyoxal concentrations (*A*) and different times with 2.5 mmol/l methylglyoxal (*B*) and then stimulated with 100 nmol/l insulin (10 min). Total cell lysates were prepared and the phosphorylation levels of PKB (on Ser^{473} and Thr^{308}) and ERK1/2 determined by Western blot analysis. *C*: The results of the densitometric analyses of three independent experiments are given (P* < 0.05; ****P* < 0.001).**

Formation of methylglyoxal-protein adducts. Methylglyoxal is a highly reactive compound capable of interfering with basic amino acids on intracellular proteins, thus affecting their structure and function (14). To investigate whether methylglyoxal interacts directly with basic amino acids of IRS proteins, the titration of free reactive amino groups was performed using the amine-reactive probe [H^3]SP (22). As shown in Fig. 8, methylglyoxal induces a pronounced reduction (~50%) in the content of free amino groups on IRS proteins. These results suggest that preincubation of L6 cells with methylglyoxal induces the formation of methylglyoxal-IRS protein adducts. Aminoguanidine is known to prevent protein modifications by dicarbonyl compounds such as methylglyoxal (24). Therefore we investigated whether pretreatment of the cells with aminoguanidine hampers methylglyoxal-IRS adduct formation and thereby restores insulin-dependent signaling pathways. Indeed, preincubation of cells with aminoguanidine, before and during treatment with methylglyoxal, results in an increase in free reactive amino groups (Fig. 8). In addition to preserving free reactive amino groups, aminoguanidine prevents the inhibition of insulin-stimulated glucose transport (Fig. 2*A* and *B*), insulin-induced phosphorylation of ERK1/2, and of PKB (Fig. 6*A–C*) when the cells are incubated for a short period with methylglyoxal. In contrast, no modification of free reactive amino groups was observed when the cells are cotreated with PDTC/methylglyoxal (Fig. 8). Consistent with this lack of modification, no protective action was observed in insulin-stimulated glucose transport (Fig. 2*A* and *B*) and in insulin-stimulated phosphorylation of ERK 1/2 and PKB (Fig. 7*A–C*). To summarize, our results confirm that the alterations in insulin-stimulated signaling pathways are not the consequence of a general methylglyoxal-induced oxidative stress or cytotoxicity and suggest that the inhibitory actions exerted by methylglyoxal in insulin signaling are essentially related to modifications at the level of IRS proteins. Consistent with this view, these inhibitory effects of methylglyoxal can be prevented by aminoguanidine.

DISCUSSION

Insulin-stimulated signaling pathways are implicated in a multitude of cellular events controling cell metabolism, growth, and differentiation. Impairment of the hormone's metabolic actions leads to insulin resistance, which is associated with several disease states such as diabetes, obesity, and atherosclerosis. A general consensus exists concerning the correlation between chronic hyperglycemia and the development of diabetic microvascular complications involved in retinopathy, nephropathy, and neuropathy (6,7). Hyperglycemia-induced tissue damage likely results from reciprocally interacting disease processes, one of which has been suggested to be the formation of AGEs. Over the last years, emerging evidence has highlighted the important role of reactive dicarbonyl AGE precursors, such as methylglyoxal, in the formation of AGEs, which in turn lead to diabetes complications (6,8). Despite the recognized implications of AGEs in generation of tissue damage in diabetes, to the best of our knowledge, no studies have been published concerning the possible impact of intracellular dicarbonyl AGE precursors on insulin resistance. Our present work shows, for the first time, that in cultured L6 skeletal muscle cells, methylglyoxal treatment affects insulin action. Indeed, a short exposure to methylglyoxal induces a dose-dependent inhibition of insulin-stimulated glucose transport in these cells.

Methylglyoxal formation is accelerated in conditions associated with hyperglycemia (13,35), and plasma methylglyoxal may be highly elevated, reaching ~0.4 mmol/l in poorly controlled type 2 diabetic patients (36). In physiological conditions, methylglyoxal is formed from dihydroxyacetone phosphate during glycolysis. Therefore, the intracellular methylglyoxal concentration is generally thought to be higher than its circulating levels. Indeed, the

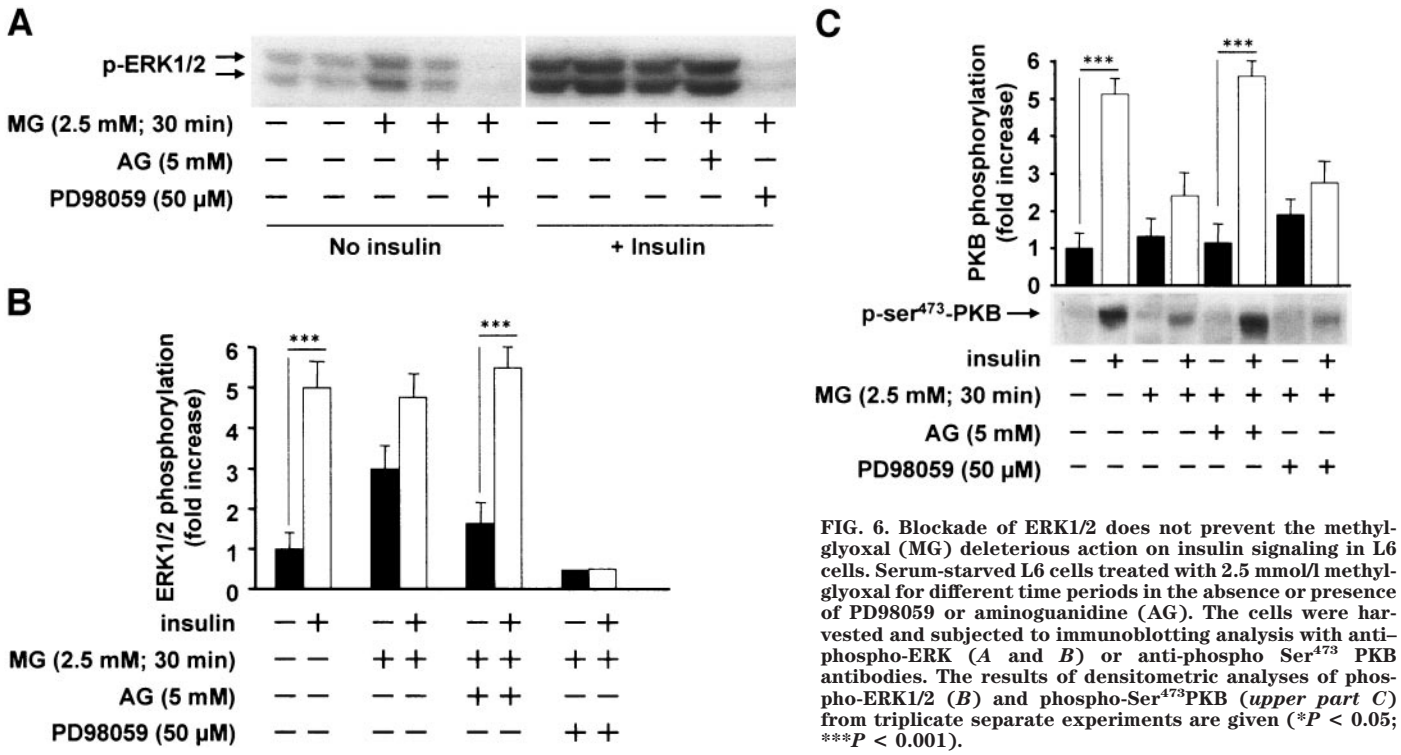

FIG. 6. Blockade of ERK1/2 does not prevent the methylglyoxal (MG) deleterious action on insulin signaling in L6 cells. Serum-starved L6 cells treated with 2.5 mmol/l methylglyoxal for different time periods in the absence or presence of PD98059 or aminoguanidine (AG). The cells were harvested and subjected to immunoblotting analysis with anti–phospho-ERK (*A* and *B*) or anti-phospho Ser^{473} PKB antibodies. The results of densitometric analyses of phospho-ERK1/2 (*B*) and phospho-Ser^{473}PKB (*upper part C*) from triplicate separate experiments are given (*$P < 0.05$; ***$P < 0.001$).

methylglyoxal level in rat aortic tissue has been reported to be about sixfold higher than in plasma (37). The intracellular concentration of methylglyoxal (free and bound) in Chinese hamster ovary cells (CHO cells) was found to be ~0.3 mmol/l (38). Considering that only a few percent (~3%) of the exogenously added methylglyoxal was taken up by our cultured L6 cells (Fig. 1), the estimated cellular concentrations of methylglyoxal implicated in the present experiments are likely to be close to the methylglyoxal levels observed in human pathological conditions (36). Moreover, the methylglyoxal concentrations utilized in our experiments have previously been used by several investigators to study the biological effects of this dicarbonyl compound (29,39–42), and they are lower than those used in vitro for physiological range AGEs production (43,44).

Methylglyoxal is known to interact with several cellular proteins and nucleic acids, thereby leading to cytotoxic effects (25,45). The cytotoxic action occurs mainly through the induction of ROS, and methylglyoxal can increase oxidative stress by inactivating glutathion reductase (46). Therefore, the alterations in insulin-stimulated signaling pathways that we observed after methylglyoxal treatment could be due to the molecular modifications induced by methylglyoxal and to the consequences of ROS production and/or methylglyoxal cytotoxicity. However, at the concentration used and over the timeframe of our experiments, no significant cytotoxic, necrotic, or apoptotic events attributable to methylglyoxal were found (Table 1). These results are in agreement with the observations previously described in isolated rat hepatocytes (28) and in human endothelial ECV304 cells (29) treated with methylglyoxal in the same concentration range.

Short exposure of L6 cells to methylglyoxal has no effect on the insulin receptor protein level or on the insulin-induced receptor tyrosine phosphorylation. This differs from the action of methylglyoxal on the epidermal growth factor (EGF) receptor in human endothelial ECV304 cells. The EGF receptor constitutes a direct target for methylglyoxal, which promotes a time- and dose-dependent inhibition of EGF signaling (29). For the deleterious action of methylglyoxal on the insulin signaling pathway, the decrease in tyrosine-phosphorylated IRS proteins was not due to impaired IRS synthesis/degradation as, after this short methylglyoxal treatment, IRS-1 protein levels were unchanged. Further, the reduction in IRS-1 tyrosine phosphorylation is not likely to be due to increased tyrosine dephosphorylation, as previous studies have reported a strong inhibition of phosphotyrosine phosphatases by α-ketoaldehydes (29,47). The decrease in insulin-stimulated phosphorylation of IRS-1 and IRS-2 (data not shown) on tyrosine was associated with a reduction in p85–IRS-1 association, PI3K activity, PKB activation, and hormone stimulation of glucose transport. All of these biological responses are prevented only by preincubating the cells with aminoguanidine, a blocker of carbonyl groups. After having established that methylglyoxal leads to impaired insulin signaling and glucose transport in L6 muscle cells, we examined the mechanisms by which this α-ketoaldehyde could act. Treatment of the L6 cells with 2.5 mmol/l methylglyoxal for 30 min induces ROS production that is blocked by the antioxidant PDTC. This ROS production is also prevented by aminoguanidine, which actually scavenges methylglyoxal and by doing so blocks its action. Using antioxidants, we were able to show that the stimulating action of methylglyoxal on ERK1/2 is due to ROS production. In contrast, no reversion of the methylglyoxal-induced inhibition of insulin-stimulated PKB phosphorylation was observed with the different antioxidants. Importantly, aminoguanidine blocks the effects of methylglyoxal on the insulin-stimulated signaling pathways, i.e., blockade of PKB phosphorylation and glucose transport. Taken together, our results provide evidence that methylglyoxal-induced impaired insulin signaling is not the consequence of increased ROS production.

Our demonstration that methylglyoxal binds directly to

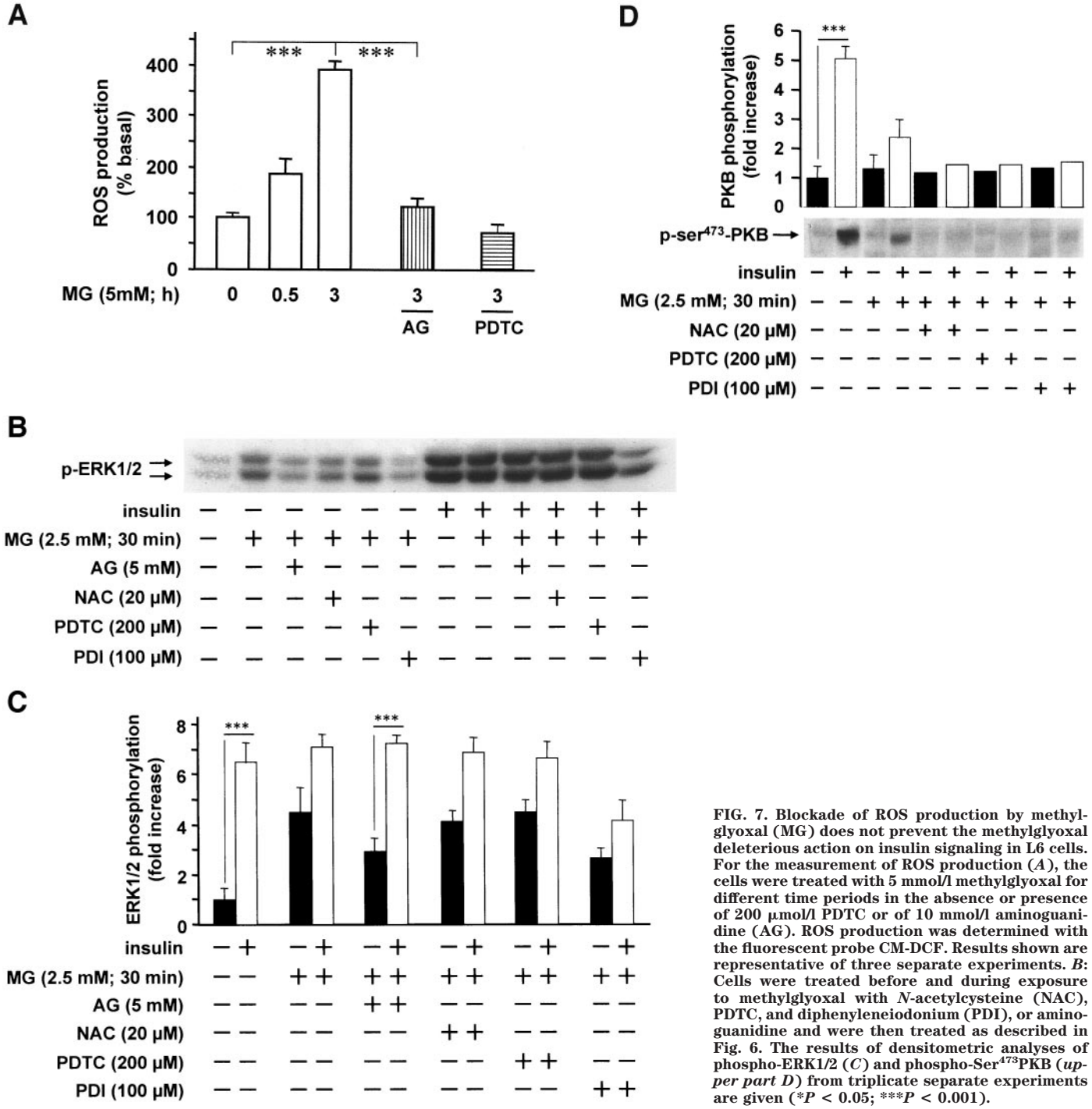

FIG. 7. Blockade of ROS production by methylglyoxal (MG) does not prevent the methylglyoxal deleterious action on insulin signaling in L6 cells. For the measurement of ROS production (*A*), the cells were treated with 5 mmol/l methylglyoxal for different time periods in the absence or presence of 200 μmol/l PDTC or of 10 mmol/l aminoguanidine (AG). ROS production was determined with the fluorescent probe CM-DCF. Results shown are representative of three separate experiments. *B*: Cells were treated before and during exposure to methylglyoxal with *N*-acetylcysteine (NAC), PDTC, and diphenyleneiodonium (PDI), or aminoguanidine and were then treated as described in Fig. 6. The results of densitometric analyses of phospho-ERK1/2 (*C*) and phospho-Ser^{473}PKB (*upper part D*) from triplicate separate experiments are given (*$P < 0.05$; ***$P < 0.001$).

IRS molecules supports the idea that the deleterious effects of methylglyoxal are due to a conformational change affecting the docking function of IRS proteins. Such a scenario could indeed explain the reduced association between PI3K and IRS that we observed. The blocking effects of aminoguanidine on these methylglyoxal-produced alterations are likely to be due to the chemical trapping of methylglyoxal. Our interpretation is in line with recent works reporting that dicarbonyl compounds, such as methylglyoxal, easily react with intracellular proteins resulting in intracellular AGE formation, which is associated with alterations of protein structure and function (48,49). By doing so, methylglyoxal affects cellular responses (46,49). It should be mentioned that it is likely that methylglyoxal interferes with additional actors in the insulin signaling pathway. However, IRS could be somehow a preferential target as it is a rather large protein with a slow turnover rate (50).

Serine phosphorylation of IRS proteins is a well-documented mechanism leading to the reduced function of these docking proteins (31). Our data do not support a role for such a mechanism in the deleterious action of methylglyoxal on insulin signaling, as we did not find increased serine phosphorylation of either IRS-1 or IRS-2 (data not shown). It should be mentioned that these data imply that the binding of methylglyoxal does not transform the IRS proteins in such a way that their phosphorylation by serine kinases is facilitated. This could have explained how the

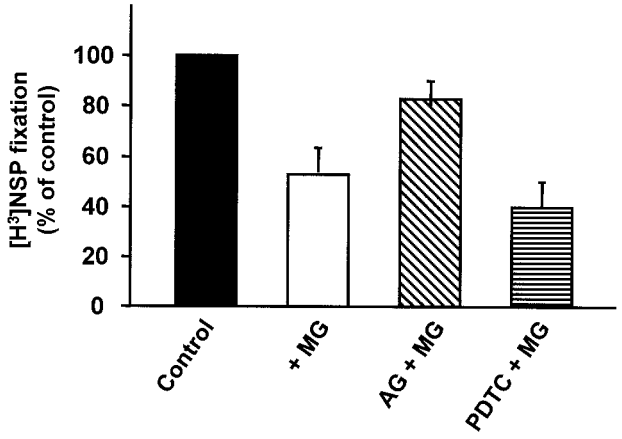

FIG. 8. Derivatization by methylglyoxal (MG) of free amino groups on IRS-1. L6 cells were left untreated (control) or were treated with methylglyoxal (5 mmol/l, 30 min) in the absence or presence of aminoguanidine (AG) (10 mmol/l) or PDTC (200 μmol/l). Thereafter, the cells were solubilized and the cell extracts subjected to immunoprecipitation with antibodies to IRS-1. Free amino groups of IRS-1 were labeled with [^{3}H]succinimidyl propionate, as indicated in RESEARCH DESIGN AND METHODS. IRS-1 was resolved by SDS-PAGE, and the [^{3}H] radioactivity associated with the molecular species corresponding to IRS-1 was counted. Results are the mean of two separate experiments.

binding of methylglyoxal decreases insulin signaling through a modification of the IRS proteins that favors serine phosphorylation.

ERK1/2 are among several kinases that have been shown to phosphorylate IRS (32,33) and are activated by ROS. However, ERK1/2 are not responsible for methylglyoxal's inhibitory effects, as we have shown that pretreatment of the L6 cells with the MEK1 inhibitor, PD98059, blocks ERK1/2 activation by methylglyoxal but does not reverse the methylglyoxal-induced inhibition of insulin-stimulated PKB phosphorylation.

To conclude, the methylglyoxal-induced alterations of IRS-1 proteins could constitute a new concept of negative regulation of insulin signaling as they appear to be essentially based on chemical modifications of IRS proteins induced by a metabolite. Further, our present work, in combination with our previous findings demonstrating that AGEs conspicuously hamper insulin's metabolic effects (19), strongly support the idea that glucose-derived products, initially thought to be involved solely in the generation of diabetes complications, also impinge in a significant fashion on insulin signaling.

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REFERENCES

1. Pessin JE, Saltiel AR: Signaling pathways in insulin action: molecular targets of insulin resistance. *J Clin Invest* 106:165–169, 2000
2. Van Obberghen E, Baron V, Delahaye L, Emanuelli B, Filippa N, Giorgetti-Peraldi S, Lebrun P, Mothe-Satney I, Peraldi P, Rocchi S, Sawka-Verhelle D, Tartare-Deckert S, Giudicelli J: Surfing the insulin signaling web. *Eur J Clin Invest* 31:966–977, 2001
3. Saltiel AR, Kahn CR: Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414:799–806, 2001
4. White MF: IRS proteins and the common path to diabetes. *Am J Physiol Endocrinol Metab* 283:E413–E422, 2002
5. Kahn BB, Flier JS: Obesity and insulin resistance. *J Clin Invest* 106:473–481, 2000
6. Brownlee M: Lilly Lecture 1993: Glycation and diabetic complications. *Diabetes* 43:836–841, 1994
7. Baynes J, Thorpe S: Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes* 48:1–9, 1999
8. Vlassara H, Palace MR: Diabetes and advanced glycation endproducts. *J Intern Med* 251:87–101, 2002
9. Degenhardt TP, Thorpe SR, Baynes JW: Chemical modification of proteins by methylglyoxal. *Cell Mol Biol* 44:1139–1145, 1998
10. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001
11. Phillips SA, Thornalley PJ: The formation of methylglyoxal from triose phosphates: investigation using a specific assay for methylglyoxal. *Eur J Biochem* 212:101–105, 1993
12. Thornalley PJ: The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. *Biochem J* 269:1–11, 1990
13. McLellan AC, Thornalley PJ, Benn J, Sonksen PH: Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. *Clin Sci* 87:21–29, 1994
14. Shinohara M, Thornalley PJ, Giardino I, Beisswenger P, Thorpe SR, Onorato J, Brownlee M: Overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis. *J Clin Invest* 101:1142–1147, 1998
15. Kim J, Kim KS, Shinn JW, Oh YS, Kim HT, Jo I, Shinn SH: The effect of antioxidants on glycated albumin-induced cytotoxicity in bovine retinal pericytes. *Biochem Biophys Res Commun* 292:1010–1016, 2002
16. Hsuuw YD, Chang CK, Chan WH, Yu JS: Curcumin prevents methylglyoxal-induced oxidative stress and apoptosis in mouse embryonic stem cells and blastocysts. *J Cell Physiol* 10: 10, 2005
17. Herrant M, Jacquel A, Marchetti S, Belhacene N, Colosetti P, Luciano F, Auberger P: Cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-induced apoptosis. *Oncogene* 23:7863–7873, 2004
18. Ceddia RB, Somwar R, Maida A, Fang X, Bikopoulos G, Sweeney G: Globular adiponectin increases GLUT4 translocation and glucose uptake but reduces glycogen synthesis in rat skeletal muscle cells. *Diabetologia* 48:132–139, 2005
19. Miele C, Riboulet A, Maitan MA, Oriente F, Romano C, Formisano P, Giudicelli J, Beguinot F, Van Obberghen E: Human glycated albumin affects glucose metabolism in L6 skeletal muscle cells by impairing insulin-induced IRS-signaling through a PKC- mediated mechanism. *J Biol Chem* 278:47376–47387, 2003
20. Germinario R, Colby-Germinario S, Cammalleri C, Wainberg M: The long-term effects of anti-retroviral protease inhibitors on sugar transport in L6 cells. *J Endocrinol* 178:449–456, 2003
21. Maziere C, Floret S, Santus R, Morliere P, Marcheux V, Maziere J-C: Impairment of the EGF signaling pathway by the oxidative stress generated with UVA. *Free Radic Biol Med* 34:629–636, 2003
22. Suc I, Meilhac O, Lajoie-Mazenc I, Vandaele J, Jurgens G, Salvayre R, Negre-Salvayre A: Activation of EGF receptor by oxidized LDL. *FASEB J* 12:665–671, 1998
23. Che W, Asahi M, Takahashi M, Kaneto H, Okado A, Higashiyama S, Taniguchi N: 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 atherogenesis in diabetes. *J Biol Chem* 272:18453–18459, 1997
24. Glomb MA, Monnier VM: Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the maillard reaction. *J Biol Chem* 270:10017–10026, 1995
25. Du J, Suzuki H, Nagase F, Akhand AA, Ma X-Y, Yokoyama T, Miyata T, Nakashima I: Superoxide-mediated early oxidation and activation of ASK1 are important for initiating methylglyoxal-induced apoptosis process. *Free Radic Biol Med* 31:469–478, 2001
26. Liu B-F, Miyata S, Hirota Y, Higo S, Miyazaki H, Fukunaga M, Hamada Y, Ueyama S, Muramoto O, Uriuhara A, Kasuga M: Methylglyoxal induces apoptosis through activation of p38 mitogen-activated protein kinase in rat mesangial cells. *Kidney Int* 63:947–957, 2003
27. Fukunaga M, Miyata S, Liu BF, Miyazaki H, Hirota Y, Higo S, Hamada Y, Ueyama S, Kasuga M: Methylglyoxal induces apoptosis through activation of p38 MAPK in rat Schwann cells. *Biochem Biophys Res Commun* 320:689–695, 2004

28. Shangari N, O'Brien PJ: The cytotoxic mechanism of glyoxal involves oxidative stress. *Biochem Pharmacol* 68:1433–1442, 2004
29. Portero-Otin M, Pamplona R, Bellmunt MJ, Ruiz MC, Prat J, Salvayre R, Negre-Salvayre A: Advanced glycation end product precursors impair epidermal growth factor receptor signaling. *Diabetes* 51:1535–1542, 2002
30. Reaven GM: Pathophysiology of insulin resistance in human disease. *Physiol Rev* 75:473–486, 1995
31. Pirola L, Johnston AM, Van Obberghen E: Modulation of insulin action. *Diabetologia* 47:170–184, 2004
32. Andreozzi F, D'Alessandris C, Federici M, Laratta E, Del Guerra S, Del Prato S, Marchetti P, Lauro R, Perticone F, Sesti G: Activation of the hexosamine pathway leads to phosphorylation of insulin receptor substrate-1 on Ser307 and Ser612 and impairs the phosphatidylinositol 3-Kinase/Akt/mammalian target of rapamycin insulin biosynthetic pathway in RIN pancreatic β-cells. *Endocrinology* 145:2845–2857, 2004
33. Bard-Chapeau EA, Hevener AL, Long S, Zhang EE, Olefsky JM, Feng GS: Deletion of Gab1 in the liver leads to enhanced glucose tolerance and improved hepatic insulin action. *Nat Med* 11:567–571, 2005
34. Potashnik R, Bloch-Damti A, Bashan N, Rudich A: IRS1 degradation and increased serine phosphorylation cannot predict the degree of metabolic insulin resistance induced by oxidative stress. *Diabetologia* 46:639–648, 2003
35. Beisswenger PJ, Howell SK, O'Dell RM, Wood ME, Touchette AD, Szwergold BS: α-Dicarbonyls increase in the postprandial period and reflect the degree of hyperglycemia. *Diabetes Care* 24:726–732, 2001
36. Lapolla A, Flamini R, Dalla Vedova A, Senesi A, Reitano R, Fedele D, Basso E, Seraglia R, Traldi P: Glyoxal and methylglyoxal levels in diabetic patients: quantitative determination by a new GC/MS method. *Clin Chem Lab Med* 41:1166–1173, 2003
37. Randell EW, Vasdev S, Gill V: Measurement of methylglyoxal in rat tissues by electrospray ionization mass spectrometry and liquid chromatography. *J Pharmacol Toxicol Methods* 51:153–157, 2005
38. Chaplen FWR, Fahl WE, Cameron DC: Evidence of high levels of methylglyoxal in cultured Chinese hamster ovary cells. *Proc Natl Acad Sci U S A* 95:5533–5538, 1998
39. Kang Y, Edwards LG, Thornalley PJ: Effect of methylglyoxal on human leukaemia 60 cell growth: Modification of DNA, G1 growth arrest and induction of apoptosis. *Leuk Res* 20:397–405, 1996
40. Biswas S, Ray M, Misra S, Dutta DP, Ray S: Selective inhibition of mitochondrial respiration and glycolysis in human leukaemic leucocytes by methylglyoxal. *Biochem J* 323:343–348, 1997
41. Paul RG, Bailey AJ: The effect of advanced glycation end-product formation upon cell-matrix interactions. *Int J Biochem Cell Biol* 31:653–660, 1999
42. Akhand AA, Hossain K, Kato M, Miyata T, Du J, Suzuki H, Kurokawa K, Nakashima I: Glyoxal and methylglyoxal induce aggregation and inactivation of ERK in human endothelial cells. *Free Radic Biol Med* 31:1228–1235, 2001
43. Ahmed MU, Brinkmann Frye E, Degenhardt TP, Thorpe SR, Baynes JW: N-epsilon-(carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem J* 324:565–570, 1997
44. Frye EB, Degenhardt TP, Thorpe SR, Baynes JW: Role of the Maillard reaction in aging of tissue proteins: advanced glycation end product-dependent increase in imidazolium cross-links in human lens proteins. *J Biol Chem* 273:18714–18719, 1998
45. Murata-Kamiya N, Kamiya H: Methylglyoxal, an endogenous aldehyde, crosslinks DNA polymerase and the substrate DNA. *Nucleic Acid Res* 29:3433–3438, 2001
46. Vander Jagt DL, Hunsaker LA, Vander Jagt TJ, Gomez MS, Gonzales DM, Deck LM, Royer RE: Inactivation of glutathione reductase by 4-hydroxynonenal and other endogenous aldehydes. *Biochem Pharmacol* 53:1133–1140, 1997
47. Zhang ZY, Davis JP, Van Etten RL: Covalent modification and active site-directed inactivation of a low molecular weight phosphotyrosyl protein phosphatase. *Biochemistry* 31:1701–1711, 1992
48. Lee HJ, Howell SK, Sanford RJ, Beisswenger PJ: Methylglyoxal can modify GAPDH activity and structure. *Ann N Y Acad Sci* 1043:135–145, 2005
49. Rosca MG, Mustata TG, Kinter MT, Ozdemir AM, Kern TS, Szweda LI, Brownlee M, Monnier VM, Weiss MF: Glycation of mitochondrial proteins from diabetic rat kidney is associated with excess superoxide formation. *Am J Physiol Renal Physiol* 289:F420–F430, 2005
50. Turnbow M, Keller S, Rice K, Garner C: Dexamethasone down-regulation of insulin receptor substrate-1 in 3T3–L1 adipocytes. *J Biol Chem* 269: 2516–2520, 1994