

BCL-2 Expression or Antioxidants Prevent Hyperglycemia-induced Formation of Intracellular Advanced Glycation Endproducts in Bovine Endothelial Cells

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Abstract

Hyperglycemia rapidly induces an increase in intracellular advanced glycation end products (AGEs) in bovine endothelial cells, causing an alteration in bFGF activity (Giardino, I., D. Edelstein, and M. Brownlee. 1994. *J. Clin. Invest.* 94:110–117). Because sugar or sugar-adduct autoxidation is critical for AGE formation in vitro, we evaluated the role of reactive oxygen species (ROS) in intracellular AGE formation, using bovine aortic endothelial cells. 30 mM glucose increased intracellular ROS formation by 250% and lipid peroxidation by 330%, while not affecting ROS in the media. In cells depleted of glutathione, intracellular AGE accumulation increased linearly with ROS generation as measured by immunoblotting and the fluorescent probe DCFH (AGE 0.258–3.531 AU* mm⁵ × 10⁴ cells, DCF 57–149 mean AU, *r* = .998, *P* < .002). Deferoxamine, α-tocopherol, and dimethylsulfoxide each inhibited hyperglycemia-induced formation of both ROS and AGE. To differentiate an effect of ROS generation on AGE formation from an effect of more distal oxidative processes, GM7373 endothelial cell lines were generated that stably expressed the peroxidation-suppressing proto-oncogene bcl-2. bcl-2 had no effect on hyperglycemia-induced intracellular ROS formation. In contrast, bcl-2 expression decreased both lipid peroxidation (100% at 3 h and 29% at 168 h) and AGE formation (55% at 168 h). These data show that a ROS-dependent process plays a central role in the generation of intracellular AGEs, and that inhibition of oxidant pathways prevents intracellular AGE formation. (*J. Clin. Invest.* 1996. 97:1422–1428). Key words: diabetes • glucose • peroxidation • reactive oxygen species • antioxidants

Introduction

A causal relationship between chronic hyperglycemia and diabetic microvascular disease, long inferred from a variety of animal and clinical studies (1) has now been definitely established by data from the Diabetes Control and Complications Trial (2), a multicenter, randomized, prospective controlled clinical study (3). A relationship between chronic hyperglycemia

and diabetic microvascular disease in non-insulin dependent diabetes mellitus (NIDDM) patients is also supported by recent literature (4).

The mechanisms by which hyperglycemia causes tissue damage and the resultant clinical complication syndromes can be conceptually divided into two categories. The first of these involves rapid changes in intracellular metabolites in response to diabetic hyperglycemia. These changes can revert to normal when hyperglycemia is abolished, but their cumulative effect leads to irreversible tissue damage. Examples of this intracellular mechanistic category include increased polyol pathway flux, increased de novo diacylglycerol synthesis, and altered intracellular redox state (5–8). The second type of mechanism by which hyperglycemia causes tissue damage involves slow changes in extracellular molecules due to hyperglycemia-induced covalent modification. These changes are irreversible for the life of the extracellular molecule. The best studied example of this extracellular mechanistic category is covalent modification of proteins by advanced glycation end products (AGEs)¹ (9, 10).

AGEs have been thought to form only on long-lived extracellular macromolecules, since the rate of AGE formation from glucose is so slow that more rapidly turned over intracellular proteins would not exist long enough to accumulate them. Recently, however, it has been demonstrated that AGEs do, in fact, form on proteins in vivo. In erythrocytes, AGE-hemoglobin accounts for 0.42% of circulating hemoglobin in normal subjects and 0.75% in diabetics (11). In endothelial cells, a primary site of hyperglycemia-induced damage, increased AGE formation is even more pronounced. After only one week AGE content increases 13.8-fold in endothelial cells cultured in high glucose-containing media (12). This extremely rapid rate of AGE formation most likely reflects hyperglycemia-induced increases in intracellular sugars which are much more reactive than glucose, such as fructose, glucose-6-phosphate and glyceraldehyde-3-phosphate (13). Basic fibroblast growth factor (bFGF) is the major AGE-modified protein in endothelial cells, but other proteins are also modified. Endothelial cell cytosol mitogenic activity is reduced 70% by AGE formation when cytosolic AGE-bFGF is increased 6.1-fold.

In vitro, chelators, sulfhydryl compounds and antioxidants profoundly inhibit AGE formation (14), presumably by reducing transition metal catalysts of oxygen radical reactions and by scavenging of various free radical species. Thus, autoxidation of either free sugars or Amadori products on protein plays a major role in AGE formation in vitro. In this study, we have

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1. *Abbreviations used in this paper:* AGEs, advanced glycation endproducts; BAE, bovine aortic endothelial cells; bFGF, basic fibroblast growth factor; DCFH, dichlorofluorescein dicetate bis(acetoxymethyl); DEM, diethyl maleate; DHAB, dihydroxybenzoic acid; MDA, malondialdehyde; ROS, reactive oxygen species; SA, salicylate; TBAR, thiobarbituric acid reactivity.

evaluated the role of reactive oxygen species (ROS) in intracellular AGE formation, using bovine aortic endothelial cells and endothelial cell lines. In addition, we have investigated the effects of free radical inhibition and peroxidation suppression on intracellular AGE formation, using antioxidants and cells stably transfected with the proto-oncogene *bcl-2*.

Methods

Cell culture conditions. Bovine aortic endothelial cells (BAE cells) (passage 4–10) were a gift from Dr. El Sabban (Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York). Transformed FBAE GM 7373 cells (15) were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, NJ). Both cell types were maintained in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS), essential and nonessential amino acids and antibiotics. Confluent cells used for experiments were incubated for different times in MEM containing 0.4% FBS (growth arrest conditions) to mimic the state of endothelial cells in vivo, with either 5 or 30 mM glucose. Culture media was changed daily, and glucose concentration was measured before each change of media using a Beckman glucose analyzer. The media and all supplements were purchased from GIBCO BRL (Grand Island, NY). BAE cells were also incubated with 30 mM glucose for 168 h in the presence of either 100 μ M deferoxamine, 5 μ g/ml α -tocopherol, 100 mM dimethylsulphoxide (DMSO) or 25 U/ml catalase, all obtained from Sigma Chemical Co. (St. Louis, MO).

Fluorescent measurement of intracellular reactive oxygen species (ROS). The intracellular formation of reactive oxygen species was detected by using the fluorescent probe dichlorofluorescein diacetate bis(acetoxy-methyl) (DCFH-DA) (Molecular Probes Inc., Eugene, OR). Cells (1×10^6 /ml) were loaded with 10 μ M DCFH-DA, and analyzed by Becton Dickinson FACSCAN with excitation and emission settings of 495 and 525 nm, respectively (16).

Extracellular free radical detection. The generation of hydroxyl radicals by glucose in the extracellular media under various culture conditions was assessed by measuring the formation of dihydroxybenzoic acid (DHBA) from salicylate using HPLC with electrochemical detection (17). Briefly, complete MEM media containing either 5 or 30 mM glucose was incubated for 24 h at 37°C in the presence of 1 mM salicylate (SA) (Sigma Chemical Co.). MEM containing 0.33 mM xanthine plus 0.4 U/ml of xanthine oxidase (Sigma Chemical Co.) was used as a positive control. Salicylate concentration was simultaneously measured with fluorescence detection (300 nm excitation, 412 nm emission) and DHBA was expressed as a ratio to salicylate recovered.

Measurement of cellular lipid peroxides. The level of lipid peroxides produced in endothelial cells under various culture conditions was fluorimetrically determined by the TBAR reaction as described previously (18). TBA-reactive substances were expressed as nmol MDA equivalents/ 10^6 cells by extrapolation from external standard curves.

Induction of intracellular free radical generation. BAE cells (1×10^6 /ml) were incubated for 3 h in 30 mM glucose MEM containing 0.5, 1, and 5 mM of the sulfhydryl reagent diethyl maleate (DEM) (Sigma Chemical Co.) which depletes intracellular glutathione.

Measurement of total intracellular AGE protein. Cytosol fractions of confluent cells were prepared as described previously (19). Equal amounts of intracellular protein from cells grown under different culture conditions were dot blotted onto a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). AGEs were detected by using the monoclonal anti-AGE antibody 6D12 (20). The immunocomplexes were visualized using an ECL kit (Amersham International, Amersham, UK) and evaluated by scanning densitometry using an Ultrascan XL (LKB Bromma, Sweden). The assay was standardized by blotting different amounts of AGE-bovine serum albumin generated by incubation of BSA (1.6 grams) with glucose (3 grams) in 10

ml of 0.5 M sodium phosphate buffer (pH 7.4) for 90 d at 37°C. The assay was reproducibly linear over a range of 0.5–50 ng/ml standard AGE-BSA (21). The specific epitope recognized by monoclonal and polyclonal antibodies to AGEs are not known. The 6D12 antibody used in these experiments has been shown not to recognize the AGE structures FFI, pentosidine, pyrrolidine or free carboxymethyllysine (20, 22, and Horuichi, S., personal communication).

Generation of lipid-peroxidation-modified protein. Stable preparations of linoleic/arachidonic acid vesicles were prepared as described (23). Liposomes (10 mg/ml) and human serum albumin (2 mg/ml) (Sigma Chemical Co.) were incubated with or without 0.1 mM CuSO_4 in 100 mM sodium phosphate buffer (pH 7.2) at 37°C for 24 h (24). At the end of the incubation, the protein was dot blotted onto a nitrocellulose membrane (Schleicher & Schuell, Inc.), and immunoblotted either with monoclonal antibody specific for MDA-lysine (MDA-2 monoclonal antibody) (25), or with the 6D12 anti-AGE monoclonal antibody. The immunocomplexes were visualized using an ECL kit (Amersham International), and evaluated by scanning densitometry using an Ultrascan XL (LKB).

Generation of *bcl-2* stable transfectants. GM7373 cells were cotransfected with the plasmids pHSV**bcl-2** and pSV₇ neo (26) using lipofectin reagent (GIBCO BRL). pHSV**bcl-2** was constructed by digesting pBSh**bcl-2** (gift of Dr. Arnon Rosenthal, Genentech, San Francisco, CA) with ECORI and ligating into pHSV-Puc (27–29) digested with the same enzyme. In this vector, *bcl-2* is under the transcriptional control of the HSV immediate early (IE) 4/5 promoter. Cells were selected against G418 (400 μ g/ml), and *bcl-2* expression was demonstrated using a mouse monoclonal antibody against human *bcl-2* (Dako, Carpinteria, CA).

Results

Hyperglycemia increases intracellular ROS formation and lipid peroxidation. To evaluate the effect of increased extracellular glucose concentration on intracellular ROS formation and an index of cumulative oxidative damage, lipid peroxidation, the intracellular ROS level was determined by DCFH-DA probe oxidation and the level of lipid peroxides in BAE cells was determined by the TBARS reaction after various incubation times in 5 and 30 mM glucose (Fig. 1). Incubation in 30 mM glucose for 24 h increased intracellular ROS 2.5-fold. No significant difference in MDA equivalents/ 10^6 cells was detected when BAE cells were exposed to 5 or 30 mM glucose for 24 h (220 ± 0.05 vs. 220 ± 0.04 pmol MDA equivalents/ 10^6 cells, $P = \text{NS}$). However, in cells exposed to 30 mM glucose for 168 h, lipid peroxides were increased 3.25-fold compared with cells exposed to 5 mM glucose (220 ± 0.05 vs. 716 ± 0.06 pmol MDA equivalents/ 10^6 cells, $P < .001$), reflecting the cumulative effect of hyperglycemia-induced ROS. To exclude the possibility that autooxidation of glucose in the media was responsible for ROS generation (30, 31), extracellular reactive oxygen species were determined in both 5 and 30 mM glucose-containing media. At the time of media change (24 h), there was no increase of total DHAB level in the 30 mM glucose MEM vs. 5 mM glucose MEM (5 mM glucose: 0.083 ± 0.01 total DHAB/SA $\times 10^3$; 30 mM glucose: 0.036 ± 0.003 total DHAB/SA $\times 10^3$; xanthine-xanthine oxidase: 3.13 ± 0.4 total DHAB/SA $\times 10^3$). Thus, the oxidative damage in BAE cells exposed to 30 mM glucose is a consequence of increased intracellular ROS production.

Increasing ROS at constant glucose concentration accelerates intracellular AGE formation. To evaluate the role of ROS in intracellular AGE formation, BAE cells were variably depleted of glutathione by increasing concentrations of DEM in 5 mM glucose-containing media (32). For each concentration

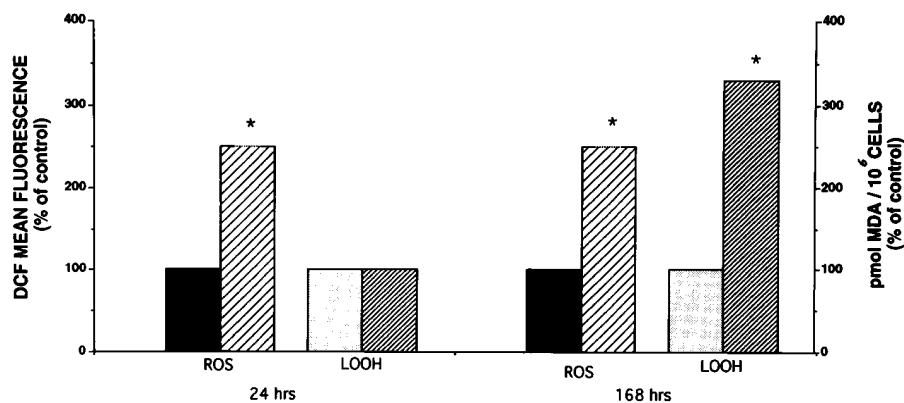


Figure 1. Effect of 30 mM glucose on BAE intracellular ROS formation and lipid peroxidation. BAE cells were incubated in media containing either 5 mM (solid bars, shaded bars) or 30 mM glucose (wide hatched bars, narrow hatched bars) for the times indicated. ROS levels were determined by FACS using the fluorescent probe DCF, and lipid peroxides were determined as TBARS. The results expressed are mean \pm SE of three experiments. * $P < 0.001$, 30 vs. 5 mM glucose.

of DEM used, both the intracellular ROS levels and the cytosol AGE-protein content were determined as described in Methods. Treatment of cells for 3 h with 0.5 mM, 1 or 5 mM DEM increased the intracellular ROS levels 1.2-, 2-, and 2.6-fold, respectively (Fig. 2). These increases in intracellular ROS were associated with a concomitant increase in intracellular AGE formation of 1.9-, 7.7- and 13.7-fold. The linear association of increased intracellular ROS formation with increased AGE formation ($r = .998$, $P < 0.002$) at constant glucose concentration suggested that a ROS-dependent process plays a central role in the generation of intracellular AGEs.

Lipid-peroxidation doesn't induce protein modification recognized by the monoclonal anti-AGE antibody. Since a number of in vitro studies suggest that lipid peroxidation and NEG/AGE formation may involve similar intermediates (33), the reactivity of the 6D12 monoclonal antibody with lipid peroxidation products was evaluated. After 24 h incubation of HSA with linoleic/arachidonic acid liposomes under oxidative conditions, the lipid peroxidation product level measured by the TBARS reaction was 3.35 ± 0.142 nmoles MDA (no MDA was detectable in the controls). Lipid-peroxide-modified proteins were detected by MDA-2 monoclonal antibody which is specific for MDA modified lysine ($AU \cdot mm \ 2.8 \pm 0.3$). Lipid per-

oxidation products were not recognized by the anti-AGE monoclonal antibody 6D12 under the same assay conditions.

Antioxidants reduce both intracellular oxidative damage and AGE formation. To evaluate the effect of ROS inhibition on intracellular AGE-modified protein formation, BAE cells were incubated for 168 h with 30 mM glucose alone or in the presence of either 100 μ M deferoxamine, 5 μ g/ml α -tocopherol, 100 mM DMSO (Fig. 3), or 25 U/ml catalase, and levels of lipid peroxides and AGEs were determined. Incubation with 30 mM glucose alone increased the level of lipid peroxides by 325% and the level of intracellular AGEs by 261%. The presence of catalase, an inhibitor of hydroxyl radical production from extracellular sources, had no effect on either intracellular lipid peroxide levels or intracellular AGE levels (data not shown). In contrast, the effects of high glucose on both intracellular lipid peroxide levels and intracellular AGE levels were completely prevented by the chelator deferoxamine, the antioxidant α -tocopherol, and the radical scavenger DMSO. These data demonstrate that there is causal relationship between hyperglycemia-induced ROS generation and intracellular AGE formation.

Bcl2 expression in endothelial cells doesn't affect the increase in ROS induced by 30 mM glucose. In lymphocytes, bcl-2

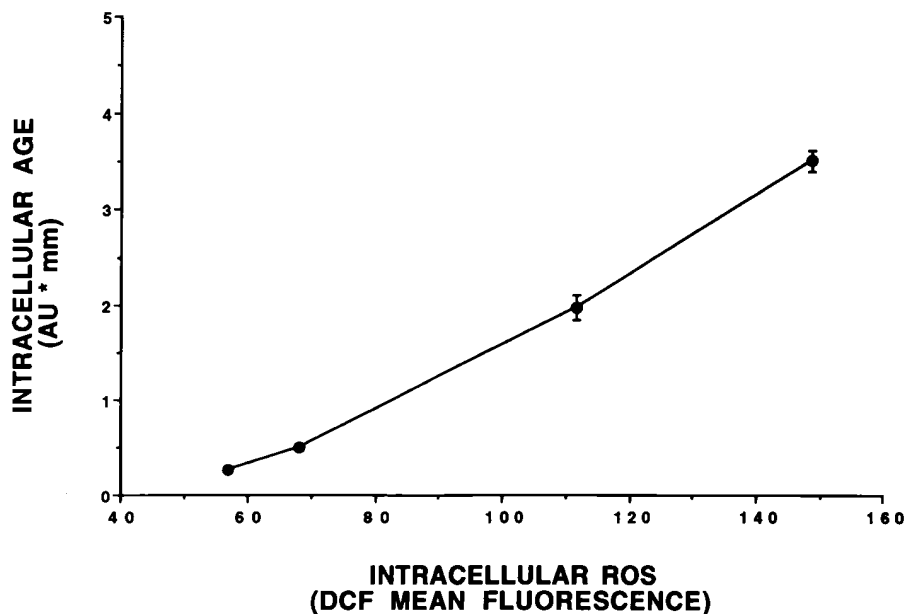


Figure 2. Effect of increasing ROS formation on intracellular AGE content. BAE cells were incubated for 3 h in media containing either 30 mM glucose and 0.5, 1.0, and 5.0 mM DEM. ROS levels were determined by FACS using the fluorescent probe DCF, and intracellular AGEs were determined scanning densitometry of immunoblots. The results expressed are mean \pm SE of three experiments.

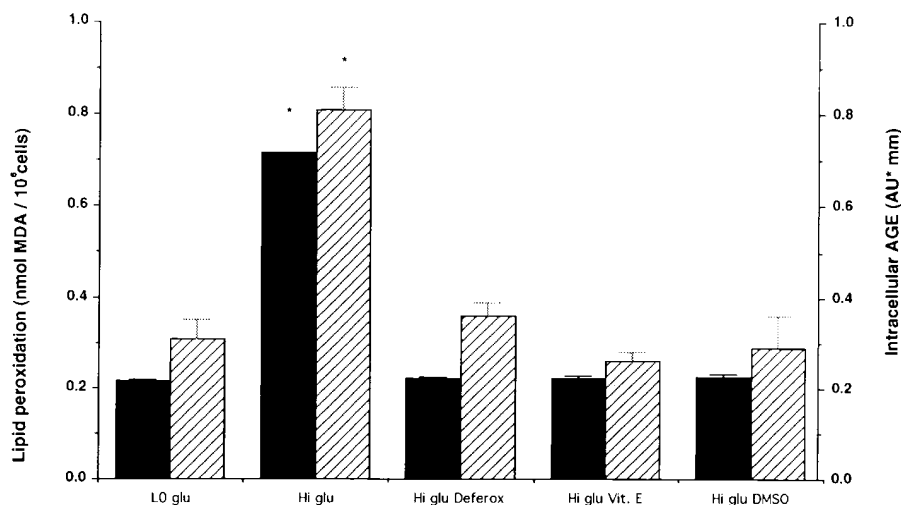


Figure 3. Effect of antioxidants on BAE intracellular ROS and AGE formation. BAE cells were incubated for 168 h in media containing either 5 mM glucose, 30 mM glucose or 30 mM glucose plus either 100 μ M deferoxamine, 5 μ g/ml α -tocopherol, or 100 mM DMSO. ROS levels (solid bars) were determined by FACS using the fluorescent probe DCF, and intracellular AGEs (hatched bars) were determined by scanning densitometry of immunoblots. The results expressed are mean \pm SE of three experiments. * P < 0.001, 30 vs. 5 mM glucose.

has been shown to prevent oxidative cell damage such as lipid peroxidation by acting at a step distal to the generation of ROS (34). Therefore, GM7373 endothelial cell lines were generated that stably expressed the peroxidation-suppressing proto-oncogene *bcl-2*, to differentiate an effect of ROS generation on AGE formation from an effect of more distal oxidative processes. GM7373 *bcl2* and GM7373 Neo cells were exposed to 30 mM glucose for different time periods and intracellular ROS levels were measured using the DCFH-DA probe. As shown in Fig. 4, no differences in the increase of free radical generation were detected between GM7373 Neo and *bcl-2* cells when they were exposed to 30 mM glucose for up to 168 h. In both cell types, after 3 and 168 h exposure to hyperglycemia, the intracellular ROS levels increased 7.3- and 11.5-fold, respectively.

Bcl2 expression in endothelial cells reduces the increase in lipid peroxidation induced by 30 mM glucose. To evaluate the effect of *bcl-2* expression on oxidative processes distal to ROS formation, the level of lipid peroxides was determined by the TBARS reaction after various incubation times in 30 mM glucose. After 3 h of incubation, lipid peroxide levels in the Neo cells were increased 1.9-fold (Fig. 5) while the levels in *bcl-2* cells were the same as at 0 time. After 168 h of incubation, lipid peroxide levels in the Neo cells were increased fivefold, while in *bcl-2* cells the hyperglycemia-induced increase was reduced by 35%.

Bcl2 expression in endothelial cells reduces the increase in intracellular AGEs induced by 30 mM glucose. To determine the effect of *bcl-2* expression on the formation of intracellular AGE-proteins, intracellular AGE content was determined in Neo and *bcl-2* endothelial cell extracts prepared after varying incubation times with 30 mM glucose (Fig. 6). After 3 h incubation, AGEs in the Neo cells were increased 3.6-fold. In *bcl-2* cells, 40% of this hyperglycemia-induced increase was prevented. Similarly, after 168 h incubation, intracellular AGEs in the Neo cells were increased 12-fold, while 61% of this hyperglycemia-induced increase was prevented in the *bcl-2* cells.

Discussion

We have previously shown that hyperglycemia rapidly increases intracellular AGE formation in bovine aortic endothelial cells, with significant functional alteration of bFGF. It was proposed that similar rapid intracellular AGE formation in susceptible diabetic tissues could contribute to the pathogenesis of vascular and neurologic complications (12). In this study, we demonstrate that hyperglycemia also increases intracellular ROS formation and subsequent lipid peroxidation in endothelial cells. When glucose concentration is held constant, increased ROS generation by DEM is associated with a concomitant increase in intracellular AGE formation. If diethylmaleate, like *N*-ethylmaleimide, also inhibited glyceraldehyde-3-phos-

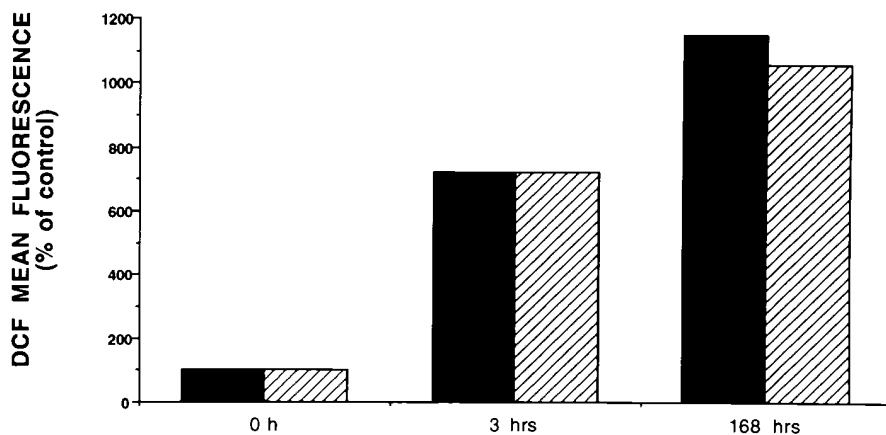


Figure 4. Effect of *bcl-2* expression on intracellular ROS formation induced by 30 mM glucose. Gm7373 endothelial cells stably transfected with either Neo (solid bars) or *Bcl-2* (hatched bars) were incubated for the indicated times in media containing 30 mM glucose. ROS levels were determined by FACS using the fluorescent probe DCF. The results expressed are mean \pm SE of three experiments. P = NS (Neo vs. *Bcl-2*) at each time point.

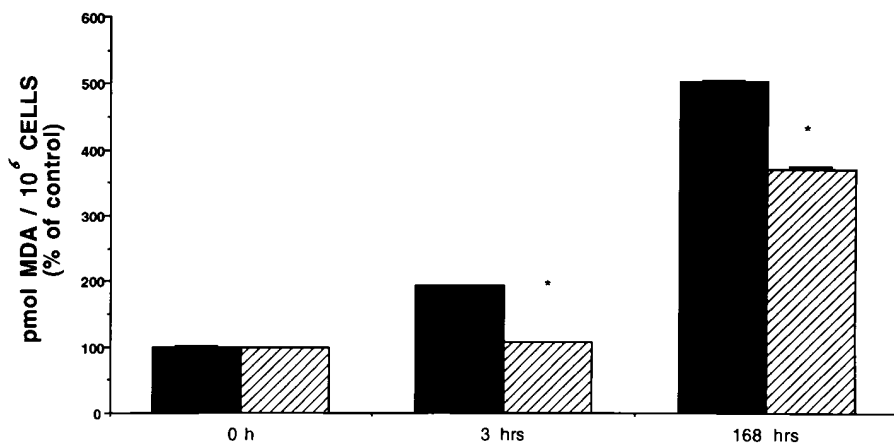


Figure 5. Effect of bcl-2 expression on lipid peroxidation induced by 30 mM glucose. Gm7373 endothelial cells stably transfected with either Neo (solid bars) or Bcl-2 (hatched bars) were incubated for the indicated times in media containing 30 mM glucose. Lipid peroxidation levels were determined as TBARS. The results expressed are mean \pm SE of three experiments. * P < 0.001, Neo vs. Bcl-2.

phate dehydrogenase (35), the concentration of AGE-forming triose-phosphates could be elevated in addition to ROS. However, no inhibition of GAPDH was observed at glutathione-depleting concentrations of DEM in mouse brain (36). ROS-induced lipid peroxidation and ROS-induced intracellular AGE formation are both completely inhibited by three different antioxidants. Expression of the peroxidation-suppressing proto-oncogene bcl-2 (34) does not effect the increase in endothelial cell ROS generation induced by hyperglycemia, but it does reduce the increase in both lipid peroxidation and AGE formation. Thus, a ROS-dependent process distal to superoxide and its conversion to peroxides plays a central role in the generation of intracellular AGEs.

Two mechanisms have been proposed that may explain how hyperglycemia causes increased ROS formation (30, 31, 37–41). One mechanism involves the transition metalcatalyzed autoxidation of protein-bound Amadori products which yields superoxide and hydroxyl radicals and highly reactive dicarbonyl compounds. The other mechanism involves the transition metal-catalyzed autoxidation of free sugars, which also yields dicarbonyl compounds and superoxide and hydroxyl radicals. Autoxidation of protein-bound Amadori products appears to be the most important source of free radicals under near physiologic conditions (40). Our findings that no ROS generation was detectable in the glucose-containing extracellular media while intracellular ROS increased 2.5-fold, and that catalase in the media had no effect on intracellular lipid peroxide levels or intracellular AGE levels, are consistent with this interpretation. It is possible, however, that inside cells exposed to hyper-

glycemia, elevated levels of non-glucose sugars (42) and non-glucose derived Amadori products make proportionately different contributions to ROS generation.

The observed association between increased ROS and intracellular AGE formation could simply reflect the fact that transition metal-catalyzed free sugar or Amadori product oxidation and free radical production are linked to the formation of dicarbonyl precursors of AGEs. Inhibition of hyperglycemia-induced lipid peroxidation and AGE formation by the iron chelator deferoxamine is consistent with this interpretation. Inhibition by α -tocopherol, a lipophilic peroxy and hydroxyl radical scavenger, and by DMSO a hydroxyl radical scavenger, however, suggests that hydroxyl radicals themselves or a more stable hydroxyl-derived radical may be directly involved in the generation of additional reactive intermediates from free sugars or Amadori products (43–46).

Further support for the hypothesis that an ROS-dependent event distal to superoxide generation and its conversion to peroxides plays a central role in the generation of AGEs comes from results of the experiments with bcl-2. This anti-apoptotic protein has been shown to block the lipid peroxidation that occurs with apoptotic cell death, while having no effect on basal or stimulated peroxide production in lymphocyte cell lines (34). Thus, bcl-2 blocks the cumulative effects of oxidative stress at a point distal to the generation of superoxide and its conversion to peroxides. In endothelial cells, bcl-2 expression has similar effects. It does not affect ROS generation induced by hyperglycemia, but it does block lipid peroxidation. At the same time, bcl-2 expression also blocks hyperglycemia-induced

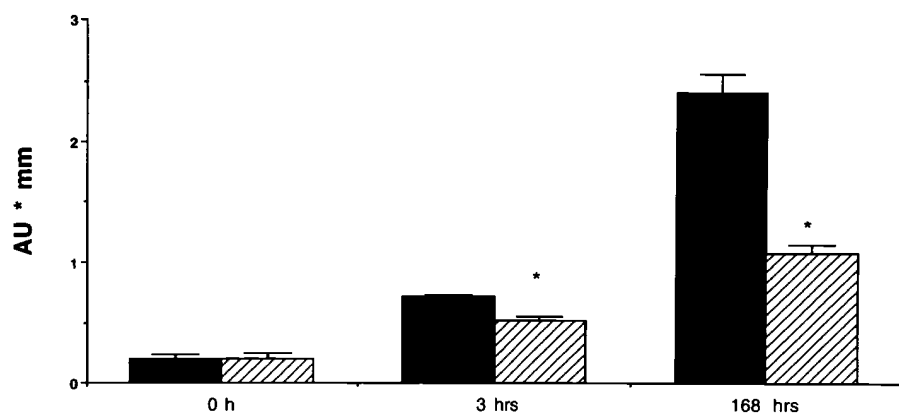


Figure 6. Effect of bcl-2 expression on intracellular AGE formation induced by 30 mM glucose. Gm7373 endothelial cells stably transfected with either Neo (solid bars) or Bcl-2 (hatched bars) were incubated for the indicated times in media containing 30 mM glucose. Intracellular AGE levels were determined by scanning densitometry of immunoblots. The results expressed are mean \pm SE of three experiments. * P < 0.001, Neo vs. Bcl-2.

AGE formation. One plausible role for bcl-2 would be as a nonreactive trap of AGE-producing free radical species. It is also possible that bcl-2 functions as a peroxidase or reductase, although such activities have not yet been detected.

The demonstration that an ROS-dependent process plays a central role in the generation of intracellular AGEs, and that inhibition of oxidant pathways prevents intracellular AGE formation have important implications for pharmacologic attempts at preventing diabetic complications. Currently, AGE formation can be reduced both by lowering hyperglycemia (47) and by blocking reactive AGE precursors with drugs such as aminoguanidine (48). Both interventions are effective in preventing diabetic complications in animal models. Blocking intracellular AGE formation by antioxidants offers an additional strategy for the potential prevention of diabetic complications that deserves further exploration.

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