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The Internalization and Metabolism of 3-Deoxyglucosone in Human Umbilical Vein Endothelial Cells

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3-Deoxyglucosone (3-DG), a dicarbonyl compound produced by glycation, plays a role in the modification and cross-linking of long-lived proteins. We synthesized [^{3}H]3-DG from [^{3}H]glucose and developed an internalization assay system using HPLC to examine its cellular metabolism. When smooth muscle cells or human umbilical vein endothelial cells were incubated with [^{3}H]3-DG, it was found that [^{3}H]3-DG was internalized by cells in a time dependent manner. The rate of internalization was reduced when the cells were incubated at 4°C or treated with phenylarsine oxide (PAO). By monitoring [^{3}H]3-DG taken up by cells, it was confirmed that 3-DG is reduced to 3-deoxyfructose (3-DF) and that this reaction was inhibited by an aldo-keto reductase inhibitor (ARI). The presence of 3-DG led to an increase in reactive oxygen species levels in the cells and subsequent apoptosis, and the effect was enhanced by pretreatment with ARI. These results suggest that 3-DG is internalized by cells and reduced to 3-DF by aldo-keto reductases, and that the internalized 3-DG is responsible for the production of intracellular oxidative stress.

Key words: aldehyde reductase, 3-deoxyglucosone, glycation, internalization.

Glycation, a non-enzymatic reaction between reducing sugars and free amino groups, has been implicated in the pathogenesis of diabetic complications and the aging process (*1*). Glycation of the °N-termianl α- and lysyl ε-amino groups of proteins by reducing sugars such as glucose leads to the formation of Schiff bases and, subsequently, Amadori products. Amadori products undergo decomposition with the formation of intermediate products such as deoxyglucosones, which ultimately react further to produce irreversible advanced glycation end products (AGEs). The pathogenesis of glycation in hyperglycemia has been studied by many groups (*2–4*); it has been proposed that the formation of Amadori products alters the function of the target proteins, the intermediate products cause the intracellular oxidative stress, and the resulting AGEs cause aggregation and insolubility of the target proteins.

3-Deoxyglucosone (3-DG), a dicarbonyl compound, has been identified as an intermediate product of glycation (*5–7*). It has been proposed that 3-DG is also produced by phosphorylation of fructose to fructose-3-phosphate and fructosamine to fructosamine-3-phosphate by fructosamine 3-kinase with subsequent decomposition (*8–12*). A number of observations indicate that dicarbonyl compounds, including 3-DG and methylglyoxal (MG), exert intracellular signaling via the production of reactive oxygen species (ROS) (*13–18*). They have been reported to induce apoptosis in U937 macrophage-derived cell lines and primary cultured cortical neurons *via* an oxidation-induced mechanism (*13*). They also induce the production of heparin-binding epidermal growth factor–like growth factor (HB-EGF) and this may be implicated in diabetic macroangiopathy (*14*). It has been shown that MG modifies and inactivates glutathione peroxidase (GPx), a central peroxide scavenging enzyme in cells (*18*). Glutathione (GSH) levels are also affected by MG treatment (*19*). Considering the fact that 3-DG causes the cross-linking of proteins and modification of their biological properties, it is likely that both MG and 3-DG increase intracellular peroxide levels by modifying antioxidative enzymes. However, details of the metabolism and intracellular effects of 3-DG have not been reported to date. In this study, we report on the cellular metabolisms of 3-DG using [^{3}H]3-DG. The results indicate that 3-DG is internalized by cells and is mainly reduced to 3-deoxyfructose (3-DF) by aldo-keto reductases. The findings herein indicate that 3-DG is responsible for the resulting intracellular oxidative stress and subsequent apoptosis.

MATERIALS AND METHODS

Materials—[^{3}H]Glucose was purchased from Amersham Pharmacia Biotech. Benzaldehyde was obtained from Nacarai Tesque (Japan). WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2-disulfophenyl)-2*H*-tetrazolium, monosodium salt] was obtained from Dojin Laboratories Co. (Japan). GSH was purchased from Sigma (St. Louis, MO). Epalrestat was obtained from Ono Pharmaceutical Co. (Japan). All other chemicals were of analytical grade.

Synthesis of 3-DG and [^{3}H]3-DG—3-DG was chemically synthesized according to the method of El-Khadem *et al.* (*20*, *21*). The structure and purity were confirmed by 1H-NMR and elemental analysis. [^{3}H]3-DG was synthesized by the same method with minor modifications,

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as follows. Synthesized 3-DG was confirmed to be endotoxin free (<1 EU/ml at 10 mM 3-DG) by using Limulus ES Test Wako (Wako Pure Chemicals , Japan).

(1) Synthesis of 3-deoxyglucosone dibenzoylhydrazone: A solution of D-glucose (17.82 mg), [^{3}H]glucose (0.18 mg), *p*-toluidine (10.7 mg), acetic acid (18.3 μl), benzoic hydrazide (27.2 mg) and 95% ethanol (0.5 ml) was refluxed at 82°C for 4 h. The reaction solution was evaporated to dryness in a stream of N_2 gas and dissolved in 1.5 ml of 2% methanol-chloroform. The solution was then charged on silica gel column (0.5 × 2 cm) and eluted with 12 ml of 2% methanol-chloroform followed by further elution with 5% methanol-chloroform. Fractions containing the 3-deoxyglucosone derivative were collected and dried on a Speed Vac concentrator (Savant). The precipitate was dissolved in 3.5% methanol-chloroform and the remaining precipitates (A). The filtrate was charged on a silica gel column (0.5 × 2 cm) and eluted with 3.5% methanol-chloroform. The fractions containing [^{3}H]3-deoxyglucosone dibenzoylhydrazone were again collected and concentrated (B). Precipitates A and B were combined (recovery = 29.8 mg, yield: 75%). Detection was performed by TLC (silica gel 60 plate, Merk) with 10% methanol-chloroform as the irrigant (R_f = 0.5).

(2) Synthesis of [3H]3-DG: A mixture of [^{3}H]3-deoxyglucosone dibenzoylhydrazone (29.8 mg), ethanol (0.9 ml), water (0.5 ml), acetic acid (35.9 μl) and benzaldehyde (45.6 μl) was refluxed for 4 h at 88°C. The reaction mixture was evaporated to dryness under a stream of N_2 gas and dissolved in 2 ml of water. The precipitate was removed by filtration and the filtrate was charged on a CHP-20 column (0.8 × 20 cm), and eluted with water. Fractions containing [^{3}H]3-DG was collected and concentrated to 1 ml (recovery = 6.5 mg, yield 54%). Detection of [^{3}H]3-DG was performed by TLC with ethyl acetate/methanol/water (5:1:1) as the irrigant (R_f = 0.4).

Cell Culture—Smooth muscle cells (SMC) were cultured in D-MEM containing 10% fetal calf serum. Human umbilical vein endothelial cells (HUVEC) were cultured in MCDB131 (Nikken Bio Med Lab, Japan) containing 10% fetal calf serum and 10 μg/ml of human basis fibroblast growth factor. Cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Throughout these experiments, the cells were used within passages 6–9.

Internalization Assay of [^{3}H]3-DG—The cells were grown to 80% confluence in 24-well plates and serum starved for 12 h. [^{3}H]3-DG (final concentration 1.25 mM) was added to each well, followed by incubation at 37°C for the indicated times. After incubation, the cells were washed three times with ice-cold D-MEM to remove unbound [^{3}H]3-DG. Cell surface bound [^{3}H]3-DG was subsequently removed by incubation with ice-cold 0.2 M acetic acid (pH 2.5) containing 0.5 M NaCl for 5 min on ice, and another short rinse with the same acidic solution. The cells were then solubilized with 500 μl of 1 N NaOH by incubation for 20 min at room temperature to quantitate the internalized [^{3}H]3-DG and its metabolites (*22*). Radioactivity was measured using a scintillation counter.

Purification of rRat Liver Aldehyde Reductase (ALR)—Aldehyde reductase was purified from rat liver as described, previously (*23*). In a typical experiment, an acetone powder of rat liver was prepared by homogenizing the tissue in acetone. After $(NH_4)_2SO_4$ fractionation (45–70%), chromatographies on DEAE-cellulose and hydroxyapatite were performed. Finally, the sample was subjected to gel filtration, and the purity of the enzyme was confirmed by SDS-PAGE.

TLC and Autoradiography—[^{3}H]3-DG and [^{3}H]3-DF were detected by TLC (silica gel 60 plate, Merk) with chloroform/methanol/water (7:3:0.3) as a solvent system and subsequent autoradiography.

Separation of 3-DG and 3-DF by HPLC—3-DG and 3-DF were separated on an Amide-80 column (Toso Co., Japan) by HPLC (SCL-10AVP, Shimadzu, Japan) at 80°C. Elution was performed at a flow rate of 1 ml/min with a mixture of 100% acetonitrile (solvent A) and water (solvent B) (0–20 min, 10–30% solvent B).

Immunocytochemistry—The cells were washed three times with phosphate-buffered saline (PBS) and then fixed for 10 min with 4% paraformaldehyde in PBS, followed by three washes with PBS. The cells were permeabilized for 5 min at room temperature with 0.2% Triton X-100 in PBS, washed twice with PBS, and blocked with 0.5% BSA in PBS for 40 min. After incubation with anti-cleaved caspase-3 for 1 h at room temperature, the cells were washed three times with PBS followed by incubation with Alexa fluor 488 goat anti–rabbit IgG (1:200, Invitrogen) and Alexa fluor 546 phalloidin (1:200, Invitorogen) in blocking buffer at room temperature for 45 min. The stained cells were washed in PBS and analyzed by confocal microscopy (Zeiss).

Western Blotting—The cells were rinsed twice with ice-cold PBS, then harvested in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% (w/v) Nonidet P-40, 10% (w/v) glycerol, 5 mM sodium pyrophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM dithiothreitol). Cell lysates were centrifuged at 15,000 × *g* for 10 min at 4°C and the supernatants were collected. The samples were subjected to 15% SDS–polyacrylamide gel electrophoresis (PAGE), and the resulting proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Germany). The blots were probed with anti-cleaved caspase-3 antibody (Cell Signaling Technology) and anti–p-42, 44 MAPK antibody (Cell Signaling Technology), then incubated with peroxidase-conjugated secondary antibody, and immunoreactive bands were visualized using an ECL kit (Amersham Pharmacia Biotech).

Cell Viability Assay—HUVEC were cultured in 96-well plates and were serum starved for 12 h. The cells were treated with 10 mM 3-DG and/or 10 μM epalrestat for 24 h. The viability of the cells was determined by using WST-1: 10 μl of WST-1 solution was added to each well, followed by incubation for 2 h at 37°C , then the absorbance was then determined at 450 nm.

Dichlorodihydrofluorecein Assay for Hydrogen Peroxide Generation—To assess the levels of intracellular peroxides, a microplate fluorescence reader assay was carried out using an oxidation-sensitive fluorescent probe, H_2DCF-DA. HUVEC were pre-incubated with or without 10 μM epalrestat for 60 min, then treated with 5 mM 3-DG for 60 min. A peroxide-sensitive dye, H_2DCF-DA, was added during the final 30 min of treatment. The fluorescence in

each well was measured with a microplate fluorescence reader using excitation and emission wavelengths of 485 and 530 nm, respectively.

RESULTS

Cellular Internalization of [^{3}H]3-DG by SMC or HUVEC—To examine the cellular metabolism of [^{3}H]3-DG, the cells were incubated with [^{3}H]3-DG at 37°C and the amounts of [^{3}H]3-DG internalized were determined. [^{3}H]3-DG was internalized into the cells in a time-dependent manner (Fig. 1, A–C). When the cells were incubated at 4°C, the internalization of 3-DG was significantly suppressed (Fig. 1A). When the cells were incubated with 10 μM phenylarsine oxide (PAO), an inhibitor of protein-tyrosine phosphatase, 3-DG internalization was also inhibited (Fig. 1, B and C). These are generally considered to be characteristic properties of endocytosis (*24*). Next, we examined the effect of glucose and fructose on 3-DG internalization. The results indicate that 3-DG uptake was not significantly affected by these sugars (data not shown), suggesting that glucose transporters are not involved in the internalization of 3-DG.

Establishment of Detection System of Internalized [^{3}H]3-DG and [^{3}H]3-DF by Use of an Amide-80 Column—Although the cellular metabolism of 3-DG has not been fully elucidated, we assumed that internalized

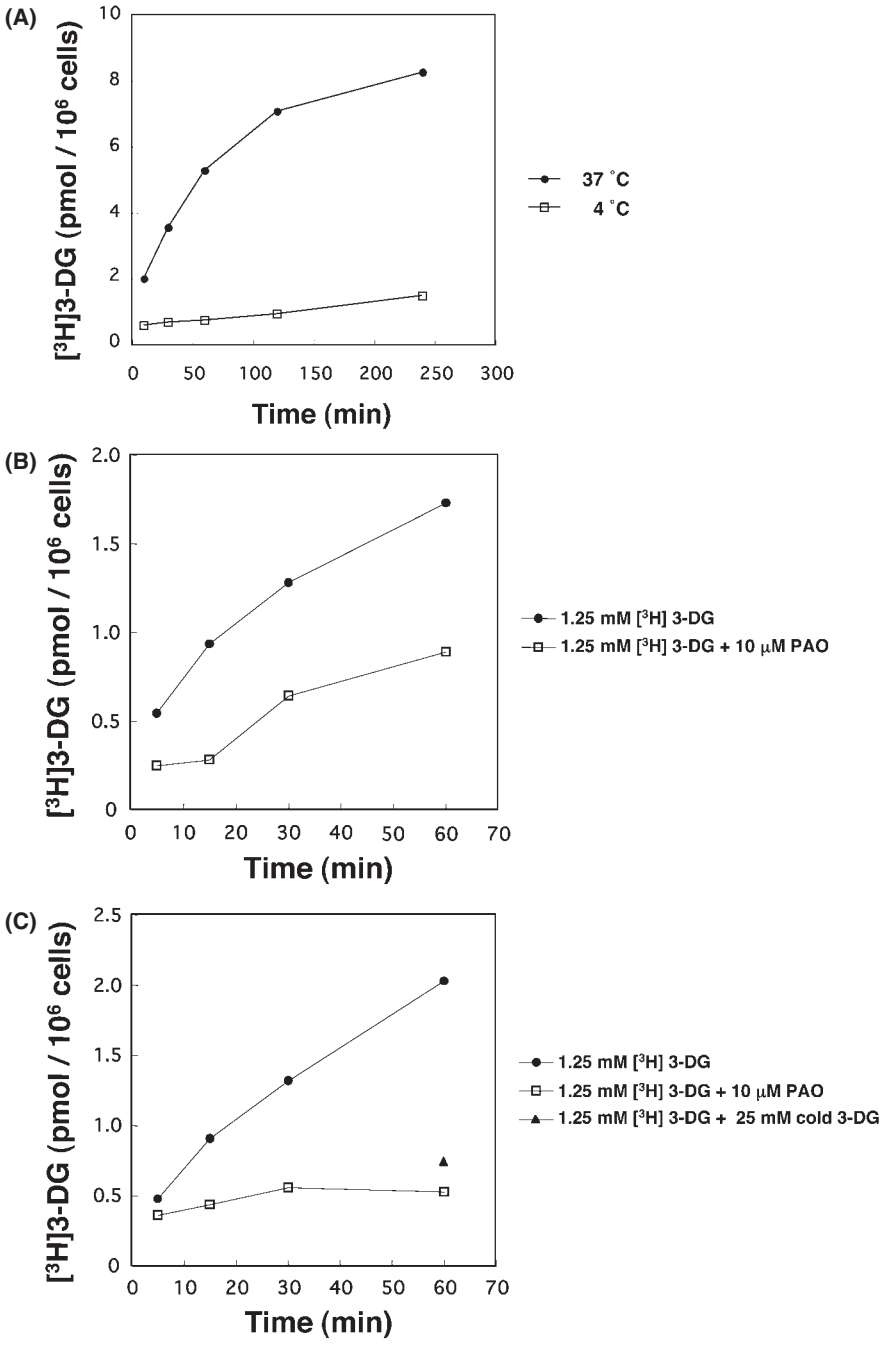


Fig. 1. **Cellular internalization of [^{3}H]3-DG by SMC or HUVEC and inhibition of the internalization.** (A) SMC were cultured in 24-well plates and serum-starved for 12 h. Then 1.25 mM [^{3}H]3-DG was added and cells were incubated at 37°C (solid circles) or at 4°C (open squares) for the indicated times. The internalized radioactivity was determined by alkaline lysis, as described under "MATERIALS AND METHODS." The figure shows one representative experiment of three independent experiments. (B) SMC were incubated with 1.25 mM [^{3}H]3-DG in the presence (open squares) or absence of 10 μM PAO (solid circles). Other conditions were the same as in (A). The figure shows one representative experiment of three independent experiments. (C) HUVEC were treated with 1.25 mM [^{3}H]3-DG in the presence (open squares) or absence of 10 μM PAO (solid circles) as in (B). Cold inhibition was examined by incubating the cells with 1.25 mM [^{3}H]3-DG and 25 mM cold 3-DG (solid triangles). The figure shows one representative experiment of three independent experiments.

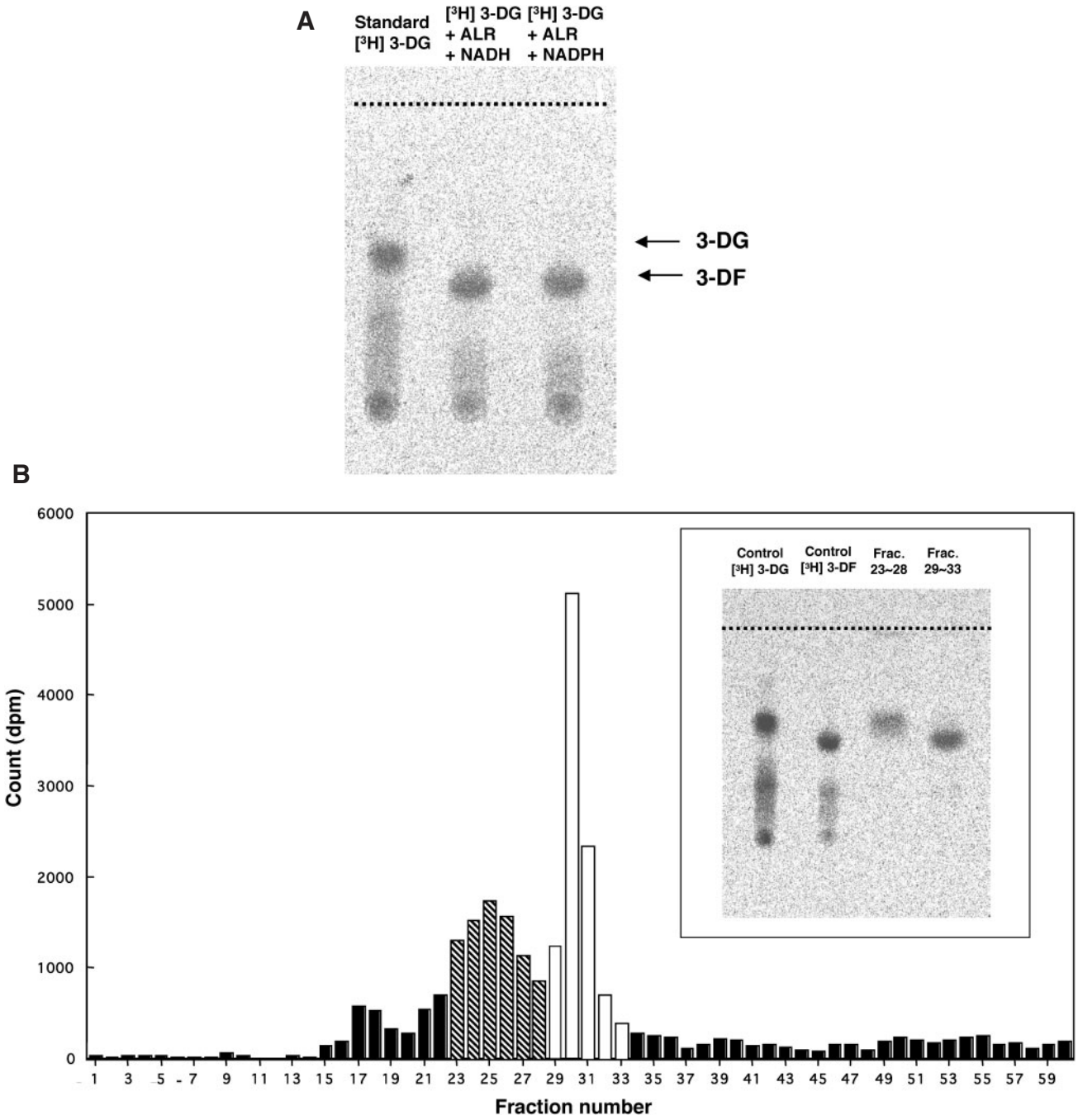


Fig. 2. **Detection of [^{3}H]3-DG and [^{3}H]3-DF by use of an Amide-80 column.** (A) TLC profile of [^{3}H]3-DG and [^{3}H]3-DF. [^{3}H]3-DF was prepared by incubating 10 mM [^{3}H]3-DG with 0.34 mg of ALR and 1 mM NADPH or NADH at 37°C for 60 min. Detection of [^{3}H]3-DG and [^{3}H]3-DF were detected by TLC with chloroform/methanol/water (7:3:0.3) as a solvent system (R_f = 0.4 and 0.36, respectively) and subsequent autoradiography. The broken line indicates the solvent front. (B) Separation of [^{3}H]3-DG and [^{3}H]3-DF by HPLC. [^{3}H]3-DG/[^{3}H]3-DF = 1:1 mixture were subjected to HPLC on Amide-80 column. Elution was performed at a flow rate of 1 ml/min with a mixture of 100% acetonitrile (solvent A) and water (solvent B) (0–20 min, 10–30% solvent B). The fraction size was 0.5 ml. (Inset) Confirmation of [^{3}H]3-DG fraction and [^{3}H]3-DF fraction of (B) by TLC with chloroform/methanol/water (7:3:0.3) as a solvent system and subsequent autoradiography. The broken line indicates the solvent front.

3-DG would be reduced to 3-DF, since aldehyde reductase, which has been shown to reduce 3-DG, is widely distributed in various tissues. [^{3}H]3-DF was synthesized by the incubation of [^{3}H]3-DG with purified rat liver aldehyde reductase (Fig. 2A). Figure 2B indicates the separation of a 1:1 mixture of [^{3}H]3-DG and [^{3}H]3-DF by HPLC. Fractions 23 to 28 (11 min to 14 min) were [^{3}H]3-DG, and fractions 29 to 31 (14 min to 15.5 min) were [^{3}H]3-DF as judged by TLC analysis (Fig. 2B, inset). These results indicate that [^{3}H]3-DG and [^{3}H]3-DF could be separated and that the amount of each can be estimated by radiochemical analysis.

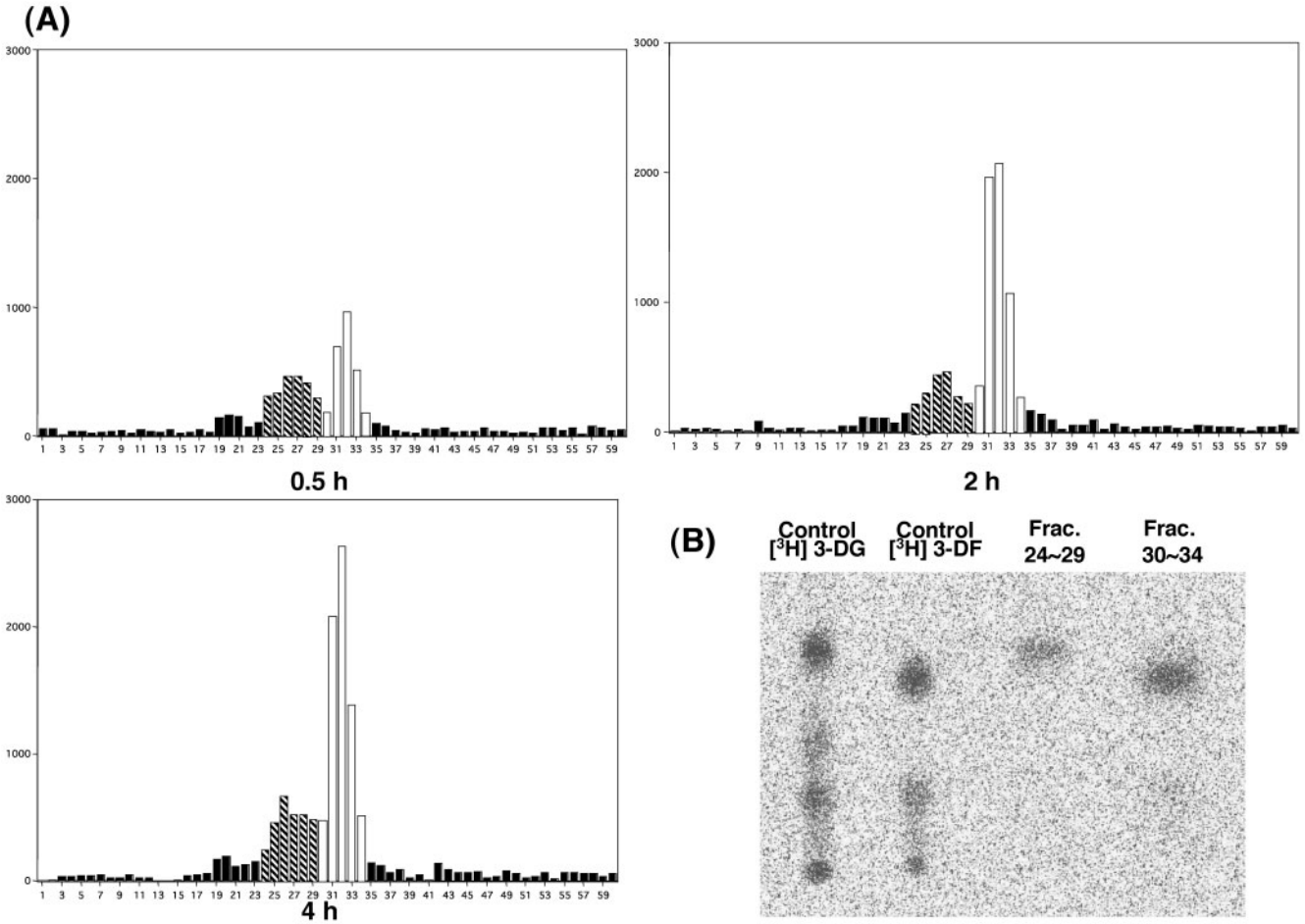


Fig. 3. **Separation of intracellular [^{3}H]3-DG and [^{3}H]3-DF by an Amide-80 column.** (A) HUVEC were serum-starved for 12 h, incubated with 1.25 mM [^{3}H]3-DG at 37°C for 0.5, 2, 4 h, and the cell extract was deproteinated by use of a Centricon YM-10 and subjected to HPLC on an Amide-80 column. Elution was performed in the same manner as described in Fig. 2. Solid squares represents [^{3}H]3-DG and open squares represents [^{3}H]3-DF. (B) Confirmation of [^{3}H]3-DG and [^{3}H]3-DF by TLC with chloroform/methanol/water (7:3:0.3) as a solvent system and subsequent autoradiography.

Internalization and Reduction of 3-DG by HUVEC—HUVEC were incubated with 1.25 mM [^{3}H]3-DG at 37°C for 0.5, 2 and 4 h. The cell extract was deproteinated by use of a Centricon YM-10 and analyzed on an Amide-80 column. As shown in Fig. 3A, [^{3}H]3-DG was reduced to [^{3}H]3-DF in a time-dependent manner. The data show that 3-DG is internalized into the cells and then reduced to 3-DF. Figure 3B indicates the confirmation of [^{3}H]3-DG and [^{3}H]3-DF fractions by TLC.

The Effect of aldo-keto Reductase Inhibitor on Cellular Metabolism of 3-DG—To identify the enzyme involved in the reduction of 3-DG to 3-DF, HUVEC were pre-incubated with or without 10 μM epalrestat (*25*) as an aldo-keto reductase inhibitor (ARI) at 37°C for 60 min and then incubated with 1.25 mM [^{3}H]3-DG for 60 min. The amount of [^{3}H]3-DF was significantly decreased by treatment with epalrestat (Fig. 4). This result suggests that [^{3}H]3-DG is reduced to 3-DF by aldo-keto reductase(s).

The Effect of 3-DG on HUVEC—To determine the effect of 3-DG and its metabolite on the cells, HUVEC were incubated with 3-DG and the apoptotic changes were examined. As shown in Figure 5A, it appears that caspase-3 was activated in the cells treated with 3-DG. Cleaved caspase-3 in 3-DG treated cells was also detected by Western blotting (Fig. 5B), indicating that 3-DG causes apoptotic cell death as previously reported (*13*). When the cells were treated with 10 mM 3-DG and 10 μM epalrestat, the extent of cell death was greater than in cells treated with either of them individually (Fig. 5C). We also examined the cytotoxicity of 3-DG by using WST-1 and found that cell death was increased in the presence of epalrestat (Fig. 5D), suggesting that aldo-keto reductase(s) detoxify 3-DG. Intracellular peroxide production was examined using a peroxide-sensitive fluorescent probe (Fig. 6). The results suggest that treatment of the cells with 3-DG and ARI produced more peroxide than treatment with either of them individually.

DISCUSSION

The non-enzymatic reaction between reducing sugars and free amino residue leads to the reversible formation of Schiff bases, which rearrange to Amadori products and ultimately to the irreversible formation of AGEs. Intermediate products such as dicarbonyl compounds have been reported to play a role in the development of diabetic

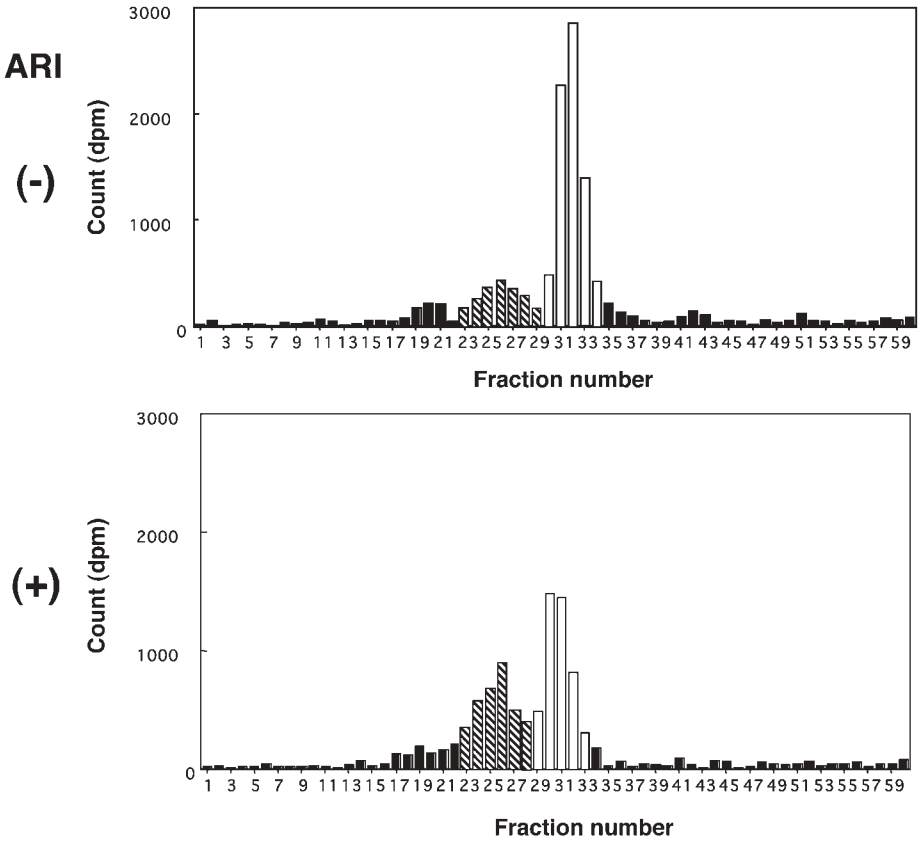


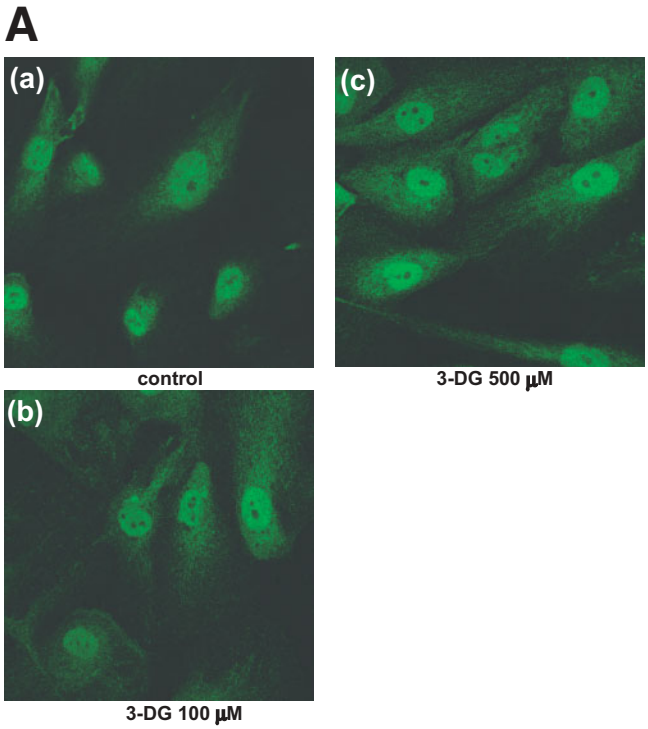
Fig. 4. **Effect of ARI on cellular metabolism of 3-DG.** HUVEC were serum-starved for 12 h, pre-incubated with or without 10 μM epalrestat (ARI) at 37°C for 60 min, then incubated with 1.25 mM [^{3}H]3-DG for 60 min. Cell extract was deproteinated by use of Centricon YM-10 and applied to an Amide-80 column. Elution was performed as described in Fig. 2. Solid squares represents [^{3}H]3-DG and open squares represents [^{3}H]3-DF, respectively.

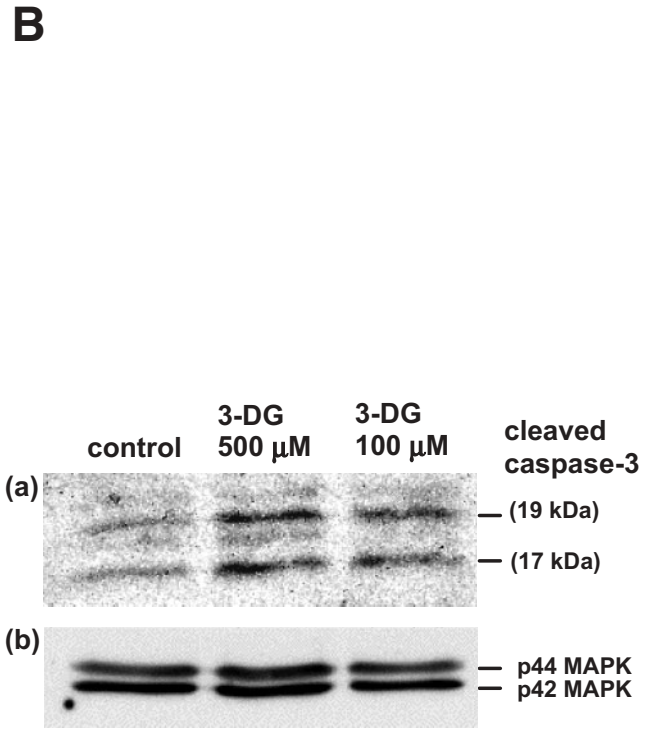
complications. However, the cellular metabolism of the intermediate products has not been clearly elucidated. We synthesized [^{3}H]3-DG and found that it is internalized into the cells. We also established a method for detecting [^{3}H]3-DG and [^{3}H]3-DF by HPLC and demonstrated that the internalized 3-DG is mainly reduced to 3-DF by aldo-keto reductase(s). When the cells were treated with 3-DG along with ARI, intracellular levels of 3-DG increased, and parallel increases in intracellular peroxide levels and apoptosis were observed. These results suggest that internalized 3-DG is involved in the increased intracellular oxidative stress.

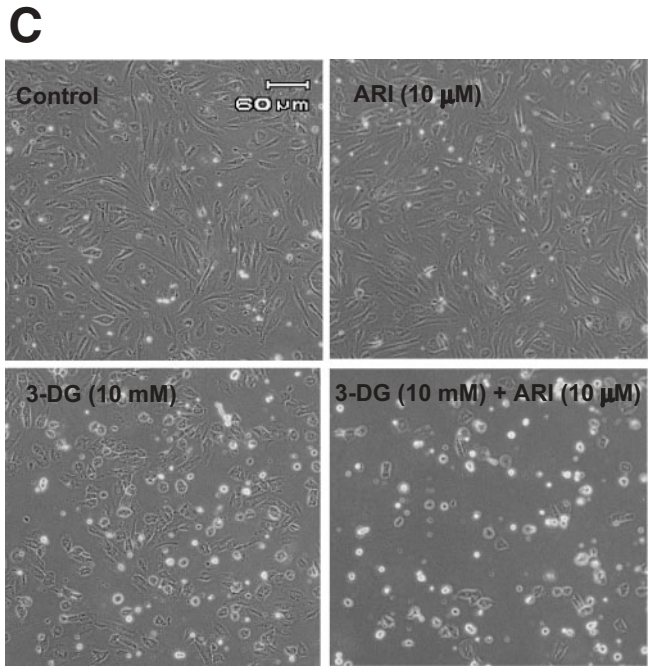
The internalization was inhibited when cells were incubated at 4°C or treated with PAO, suggesting that 3-DG is, at least in part, internalized by endocytic uptake. We hypothesized that glucose transporters are involved in the internalization of 3-DG, but neither glucose nor fructose clearly suppressed the 3-DG uptake. We also hypothesized that a specific receptor or transporter of 3-DG might be present on the cell surface, as there are specific interaction sites for AGEs such as the macrophage scavenger receptor (*26*), galectin-3 (*27*), CD36 (*28*), and the receptor for AGE (29), but no significant evidence was observed. Thus, details of the mechanism and role of 3-DG internalization remain unclear.

Several pathways for the metabolism of 3-DG have been proposed. Kato *et al.* reported that the intravenous administration of [^{14}C]3-DG to rats resulted in the excretion of [^{14}C]3-DF in urine (*5*). Kanazu et al. and our group identified aldehyde reductase as the enzyme that catalyzes the reduction of 3-DG to 3-DF (*23*, *30*). It has also been proposed that 3-DG is oxidized to 2-keto-3-deoxygluconic acid by oxoaldehyde dehydrogenase (*31*). Our results indicate that 3-DG is mainly reduced to 3-DF in HUVEC, at least in the present system, since no significant peak other than [^{3}H]3-DF was observed, as shown in Fig. 3. We examined oxoaldehyde dehydrogenase activity in HUVEC but could not detect specific activity. Our results also indicate that the conversion of intracellular 3-DG to 3-DF is catalyzed by aldo-keto reductase(s), since the reaction was suppressed when the cells were treated with ARI. Aldo-keto reductases have been shown to function as self-defense enzymes against carbonyl compounds (*32–34*). Among the aldo-keto reductase superfamily, ALR could be the main enzyme responsible for the reduction of 3-DG in most tissues, although aldose reductase (AR) has similar functions (*35*, *36*), since ALR is distributed more widely than AR. We previously reported that ALR is glycated mainly at Lys-140 and inactivated under conditions of hyperglycemia (*37*). It is possible that cellular ALR is also modified by 3-DG and inactivated, since 3-DG has been shown to be highly reactive and the Lys-140 residue is likely to be a target of carbonyl modification.

We assume that the intracellular ROS produced by 3-DG is hydrogen peroxide. Several mechanisms have been proposed to explain how intracellular ROS levels are elevated by dicarbonyl compounds. For example, (i) carbonyl compounds directly modify and inactivate antioxidative enzymes such as GPx or thioredoxin reductase (*17*, *18*); (ii) cellular levels of thiols, such as GSH, are decreased by direct modification with carbonyl compounds or by being consumed during the enzymatic detoxification of







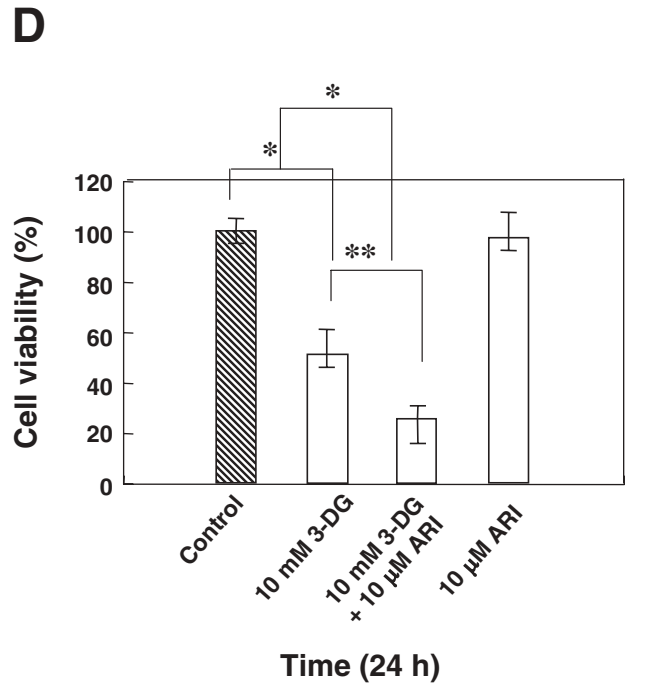


Fig. 5. **Effect of 3-DG and its metabolite on the viability of HUVEC.** (A) Expression of cleaved caspase-3 in HUVEC. The cells were cultured in 2% FCS for 12 h, then incubated without 3-DG (a), or with 100 μM 3-DG (b) or 500 μM 3-DG (c) for 72 h at 37°C. Cells stained with anti-caspase-3 antibody are shown (green). (B) Western blot analysis of cleaved caspase-3. HUVEC were cultured in 2% FCS for 12 h, then treated with 100 μM or 500 μM 3-DG for 72 h at 37°C. (a) Anti-cleaved caspase-3 antibody, (b) anti–p-42, 44 MAPK antibody. (C) HUVEC were treated with 10 mM 3-DG and/or 10 μM epalrestat (ARI) at 37°C for 12 h, and visualized by optical microscopy. Magnification ×40. The bar indicates 60 μm. (D) Effect of 3-DG on the viability of HUVEC. The cells were treated with 10 mM 3-DG after treatment of with or without ARI and subjected to viability assay using WST-1 . The assay was carried out in quadruplicate. The error bar indicates the SDs. * and ** denote significant difference at $p < 0.01$ and $p < 0.05$, respectively.

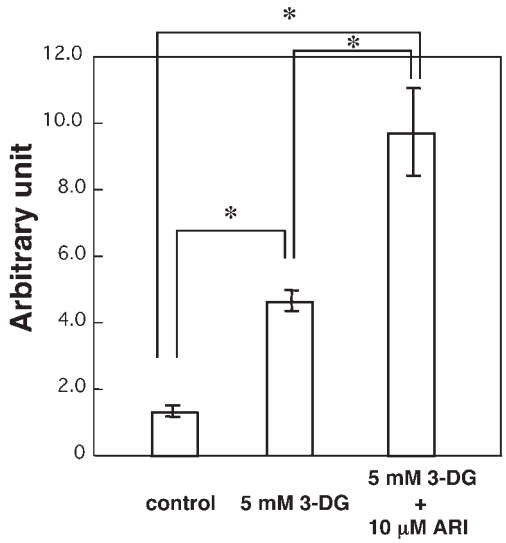


Fig. 6. **Peroxide production in HUVEC by 3-DG and/or ARI treatment.** HUVEC were pre-incubated with or without 10 μM epalrestat (ARI) for 60 min, then treated with 5 mM 3-DG for 60 min. A peroxide-sensitive dye, H_2DCF-DA was added during the final 30 min of treatment. Relative peroxide concentrations in the cells were then quantitated by use of a fluorescence plate reader. The assay were carried out in quadruplicate. The error bar indicates the SDs. * denotes significant difference at $p < 0.01$.

the carbonyl compounds (*19*); (iii) ROS are produced during protein or amino acid glycation by carbonyl compounds (*15*); and (iv) extracellular carbonyl compounds act on certain membrane bound proteins and subsequently increase the level of oxidative stress in the cell. Since 3-DG has been reported to react with lysine or arginine residues of proteins, it is likely that 3-DG modifies antioxidative enzymes, causing their inactivation. We have found that 3-DG appeared to have a greater GPx-inactivation effect than 3-DF (Sakiyama H, unpublihsed observation). Thus, it seems likely that the carbonyl residues on 3-DG play some role in ROS production. It is also considered that ROS production is a consequence of apoptosis rather than a cause of it. How ROS is produced in 3-DG treated cells is a topic for future studies.

In this study, we used a concentration of 3-DG in the order of 100 μM–10 mM, which is much higher than serum 3-DG levels in diabetes, around 400 nM (*38*). However, we observed that the internalization of 3-DG was endocytotic and appeared to proceed in an active manner, thus 3-DG may act at physiologically relevant concentrations. Moreover, the intracellular concentration of 3-DG as judged by [^{3}H]3-DG incorporation was much lower, in the range of 10–100 μM, suggesting that 3-DG produced intracellularly may act and be metabolized as proposed herein. 3-DG is also considered to increase in the microenvironment under hyperglycemic conditions, since the activity of 3-DG metabolizing enzyme would be suppressed by modification. It is noteworthy that cytosolic concentrations of 3-DG and MG are significantly increased by toxicant-treatment including oxidative stress (*39*). Eriksson et al. reported that exogenous 3-DG (100 μM) yielded an increased embryonic malformation and that 3-DG is much more toxic than MG, suggesting that metabolism of 3-DG may be important in this setting (*40*).

The current study confirms the internalization and metabolism of extracellularly administrated 3-DG in HUVEC. The results suggest a crucial relationship between the metabolism of dicarbonyl compounds and intracellular oxidative stress. 3-DG imidazolones are major 3-DG–derived AGEs in vivo (*41*) and they are found in the cytoplasm of foam cells in the atheromatous core (*42*) and in nodular lesions in diabetic nephropathy (*43*). Thus, it is suggested that intracellular 3-DG accumulation is involved in the pathogenesis of diabetes and atherosclerosis. Further investigation is needed to elucidate the detailed mechanisms by which 3-DG is internalized and peroxides are produced in cells.

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