Age-dependent cell death and the role of ATP in hydrogen peroxide-induced apoptosis and necrosis

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Contributed by Earl R. Stadtman, December 7, 2005

Cell death plays a pivotal role in the body to maintain homeostasis during aging. Studies have shown that damaged cells, which must be removed from the body, accumulate during aging. Decay of the capacity and/or control of cell death during aging is widely considered to be involved in some age-dependent diseases. We investigated the accumulation of protein carbonyls and the role of cell death induced by hydrogen peroxide in human fibroblasts from individuals of various ages (17–80 years). The results showed that levels of oxidatively modified proteins increased with age, not only in whole-cell lysates but also in mitochondrial fractions, and this change correlates with a decline in the intracellular ATP level. Exposure of fibroblasts to hydrogen peroxide led to cell death by apoptosis and necrosis. Younger (<60 years old) cells were more resistant to necrosis induced by hydrogen peroxide than were older cells (>60 years old), which contained lower levels of free ATP than did younger cells. Treatment of cells of all ages with inhibitors of ATP synthesis (oligomycin, 2,4-dinitrophenol, or 2-deoxyglucose) made them more susceptible to cell death but also led to a switch in the death mode from apoptosis to necrosis. Furthermore, hydrogen peroxide treatment led to a greater accumulation of several inflammatory cytokines (IL-6, IL-7, IL-16, and IL-17) and increased necrosis in older cells. These results suggest that age-related decline in the ATP level reduces the capacity to induce apoptosis and promotes necrotic inflammation. This switch may trigger a number of age-dependent disorders.

protein carbonyls | cytokines | oxidative stress | mitochondria

Aging is characterized by a general decline in physiological function that leads to morbidity and mortality. Although specific causes of this decline are not well known, there is some evidence that sustained damage inflicted by endogenously produced oxidants contribute to the development of the age-related deficits (1). These oxidants include superoxide ($O_2^{\bullet -}$), hydrogen peroxide, and hydroxyl radicals (HO^-) and possibly singlet oxygen ($^1O_2$). They damage cellular macromolecules, including DNA (2), proteins (3), and lipids (4). The continuous threat of oxidative damage to the cell, tissue, and organism as a whole is underscored by the existence of an impressive array of cellular defenses that have evolved to battle these reactive oxidants. However, these defenses are not perfect and, consequently, cellular macromolecules become oxidatively damaged. If a cell becomes damaged and nonfunctional, apoptosis provides a mechanism to eliminate it to make room for its replacement with a new cell. If the apoptotic process does not work well, the accumulation of damaged cells will contribute significantly to aging (5) and age-associated degenerative diseases such as cancer, cardiovascular disease, immune system decline, brain dysfunction, and cataracts.

Mitochondria constitute the greatest source of oxidants on the basis of the following evidence: (*i*) the mitochondrial electron transport system consumes ≈85% of the oxygen used by the cell, and, (*ii*) in contrast with other oxidant-producing systems of the cell (cytochrome P450, various cytosolic oxidases, β-oxidation of fatty acids in peroxisome, etc.), mitochondria are required for the production of ATP and are present in relatively high numbers in essentially all cells of the body. However, a number of studies have demonstrated that mitochondrial integrity also declines as a function of age (1). One plausible theory is that an initial reactive oxygen species-induced impairment of mitochondria leads to increased oxidants production that, in turn, leads to further mitochondrial damage. Therefore, as many studies have demonstrated, old mitochondria appear morphologically altered and produce more oxidants and less ATP (1). A decline in mitochondrial function can lead to lower ATP production and can decrease the efficiency of energy-dependent processes and ATP-mediated cell signal transduction.

At present, there is no question that apoptosis requires energy, because it is a highly regulated process involving a number of ATP-dependent steps, such as caspase activation, enzymatic hydrolysis of macromolecules, chromatin condensation, blebs formation, and apoptotic body formation (6–12). It was also found that depletion of cellular ATP by chemicals or in p^0 cells causes switching of the cell death mechanism from apoptotic cell death triggered by a variety of stimuli to necrotic cell death (13, 14).

In this study, we examined the susceptibility to the oxidative stress-induced apoptosis, especially focusing on the intracellular ATP level, in the functionally aged cell. We used hydrogen peroxide as an oxidative stress-related apoptosis inducer, because hydrogen peroxide is one of the most common endogenous oxidants. Our results show that functionally older cells with lower ATP levels were more sensitive to hydrogen peroxide-induced necrotic cell death than were younger cells (with higher ATP levels). Younger donor cells with higher ATP levels exhibit a higher threshold for hydrogen peroxide-induced apoptosis, whereas cells from older donors rapidly started to die by necrosis. We also demonstrate that necrotic cell death in the older cell enhanced inflammatory cytokine accumulation, and we discuss the relationship between ATP-dependent switching of the form of cell death in the aged cells and several age-dependent inflammatory disorders.

Results

Oxidative Modification of Proteins in Cultured Human Fibroblasts. Consistent with results of earlier studies (15), we show here that there is little or no change in the level of oxidized proteins (protein carbonyl derivatives) in cultured human fibroblasts from individuals over the range of 17–60 years of age. However, fibroblasts from older individuals (60–80 years of age) contained significantly higher (up to 1.8-fold) levels of oxidized proteins (Fig. 1). Because mitochondria are a major source of reactive oxygen species involved in protein oxidation (1), we also determined protein carbonyl levels in mitochondria fractions. Our results show that, as in whole-cell lysates, carbonyl levels in mitochondria fractions were also elevated as a function of age. This result is in agreement with earlier studies showing that

Conflict of interest statement: No conflicts declared.

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Abbreviations: OLG, oligomycin; DNP, 2,4-dinitrophenol; 2DG, 2-deoxyglucose; LDH, lactate dehydrogenase.

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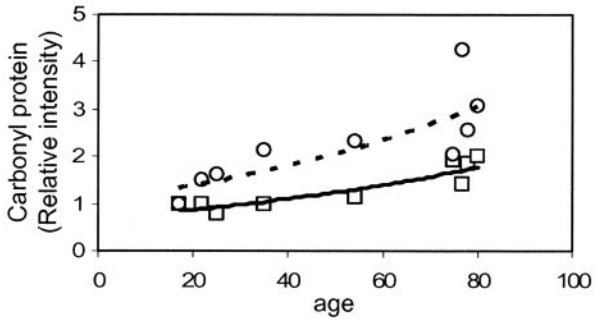

Fig. 1. Age-dependent protein oxidation in human fibroblasts. The protein carbonyl content in whole-cell lysates and mitochondrial fractions was determined by using 2,4-dinitrophenylhydrazine as described in *Materials and Methods*. □ and solid line represent whole-cell lysates, and ○ and dashed line represent mitochondrial fractions. The levels of carbonyl in both whole-cell lysates and mitochondrial fractions are normalized to a value of 1 for age-17 donors.

oxidative damage in mitochondria increases with age (16, 17). Interestingly, the rate of protein carbonyl accumulation is significantly higher in the mitochondria fraction than in the whole-cell lysate.

Age-Dependent Decline of Intracellular ATP and Hydrogen Peroxide Induced c-jun Phosphorylation. Temporary or sustained loss of mitochondrial ATP generation can have a major impact on the fidelity of cellular defenses and repair processes. Therefore, we measured the ATP concentrations of human fibroblasts from individuals of different ages. As shown in Fig. 2, the intracellular level of ATP decreased as a function of the age of the fibroblast donor.

Because hydrogen peroxide-induced phosphorylation of c-jun transcription factor by c-jun N-terminal kinase plays a critical role in signal transduction (18), we examined the effect of both cell age and hydrogen peroxide treatment on the phosphorylation of c-jun. As shown in Fig. 3*A*, the level of c-jun protein was not affected by age or by hydrogen peroxide treatment. However, the level of phosphorylated c-jun at Ser-73 decreased substantially with increasing cell age (Fig. 3*A*) and with hydrogen peroxide treatment (Fig. 3*B*). There is an inverse correlation between protein carbonyl accumulation and c-jun phosphorylation under conditions of oxidative stress.

Effect of Aging on Cell Susceptibility to Hydrogen Peroxide-Induced Cell Death. To investigate the effects of ATP and hydrogen peroxide stress on cell viability and caspase-3-like activities, we exposed cultured fibroblasts from old and young individuals to

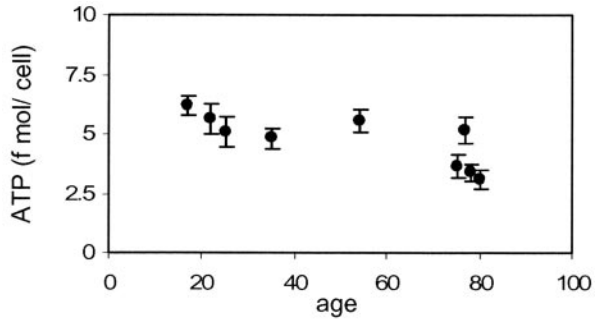

Fig. 2. Cellular ATP levels decrease as a function of age. ATP was measured as described in *Materials and Methods*. Values are the mean of three independent experiments, presented with SDs.

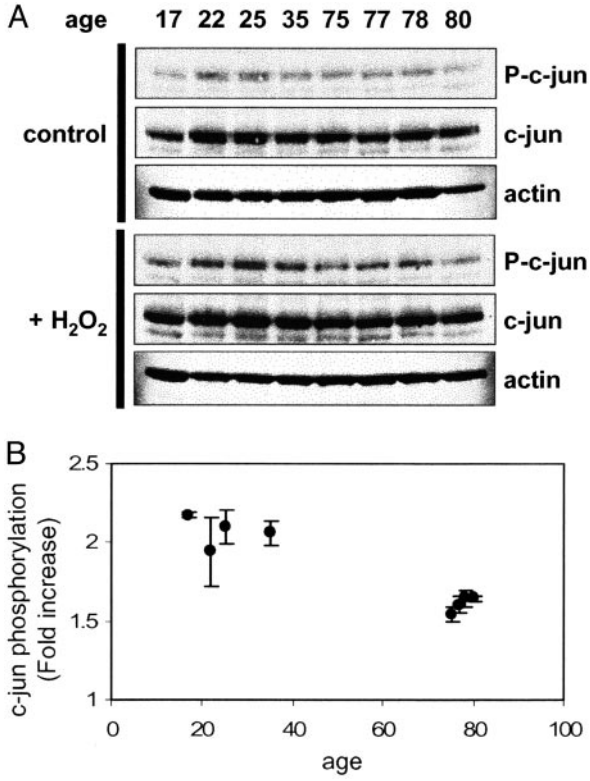

Fig. 3. Age-dependent decline in hydrogen peroxide-induced phosphorylation of c-jun. (*A*) Fibroblasts from donors of various ages were treated with or without 30 μM hydrogen peroxide for 3 h. Immunoblot analyses were performed by using anti-c-jun, anti-Ser-73 phospho-c-jun, and anti-actin antibodies. (*B*) Effect of donor's age on the ratio of phosphorylated c-jun observed in the presence of 30 μM hydrogen peroxide compared with that observed in the absence of hydrogen peroxide.

increasing concentrations of hydrogen peroxide from 0 to 40 μM in the presence or absence of inhibitors that lead to a decrease in the intracellular level of free ATP. From data summarized in Fig. 4*A*, it is evident that older cells are more sensitive than young cells to hydrogen peroxide-induced loss of viability. Exposure of the older cells to 20, 30, and 40 μM hydrogen peroxide for 16 h led to 16%, 59%, and 91% losses in cell viability, respectively. However, exposure of the younger cells to the same concentrations of hydrogen peroxide led to losses of only 7%, 25%, and 61%, respectively. Moreover, pretreatment of the cells with inhibitors that reduce the intracellular level of free ATP by 20% [2-deoxyglucose (2DG), oligomycin (OLG), or 2,4-dinitrophenol (DNP)] led to even greater losses in cell viability, except for the case of young cells treated with OLG, at all hydrogen peroxide concentrations used (Fig. 4*A*). The effect of hydrogen peroxide in the presence of ATP synthesis inhibitors was more pronounced in older cells than in younger cells.

As expected, the caspase-3-like activities of both old and young cells were also increased by treatment with 20–30 μM hydrogen peroxide (Fig. 4*B*). Treatment with 30 μM hydrogen peroxide led to 6-fold and 8.6-fold increases in caspase-3-like activity in old and young cells, respectively. By comparison, treatment with 40 μM hydrogen peroxide led to almost complete inhibition of caspase-3-like activity in the old cells, whereas activity in the young cells at 40 μM was ≈40% of that observed at 30 μM. Data summarized in Fig. 4*B* show also that hydrogen peroxide-induced caspase-3-like activation is greatly suppressed by pretreatment of cells for 1 h with 2 μM OLG, 100 μM DNP, or 200 μM 2-DG that leads to a 20% decrease in intracellular

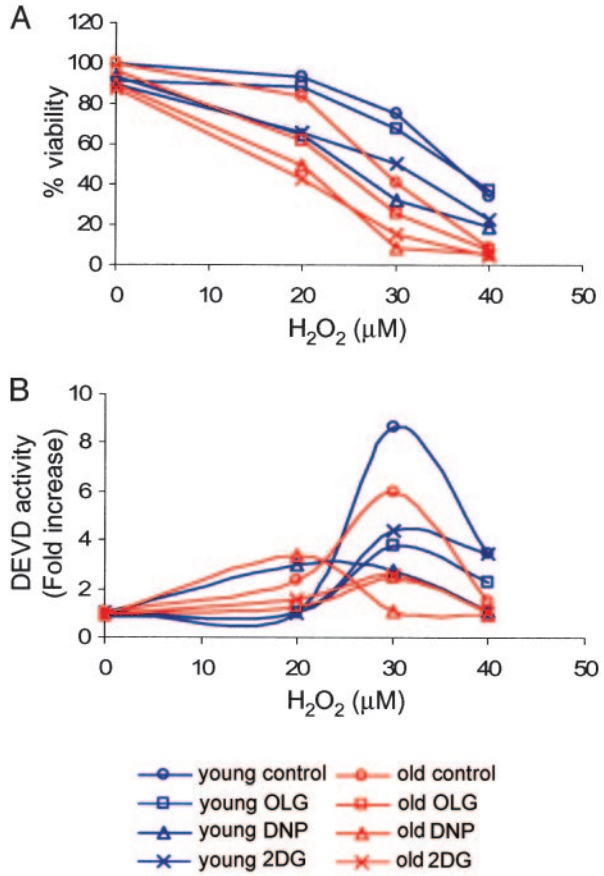

Fig. 4. Effect of ATP inhibitors on hydrogen peroxide-induced cell viability and caspase-3-like activity in young and old fibroblasts. (*A*) ATP inhibitors induce the susceptibility of young and old cells to hydrogen peroxide-induced cell death. Cell viability was determined for a mixture of young (17, 22, 25, and 35 years, blue lines) and old (75, 77, 78, and 80 years, red lines) cells. Cells were pretreated for 1 h with or without 2 μM OLG, 100 μM DNP, or 200 μM 2DG, as indicated, followed by a 16-h incubation with the indicated concentrations of hydrogen peroxide. (*B*) Caspase-3-like activity after treatment of young and old cells, as described in *A*. Values are mean of the four different ages of cells in three independent experiments.

ATP levels. But, again, the younger cells are less sensitive to hydrogen peroxide treatment in the presence of ATP synthesis inhibitors than are older cells. Interestingly, treatment with DNP also led to a shift in the concentrations of hydrogen peroxide that yielded maximum caspase-3-like activity in both old and young cells. In addition, we observed little or no change in caspase-3 and caspase-9 constitutive expression levels in either old or young cells, as indicated by Western blot analysis (data not shown). Our results demonstrate that younger cells are more resistant than are older cells to hydrogen peroxide-induced cell death. The enhanced cell death observed with older cells is associated with a decrease in the intracellular level of free ATP, a condition that favors necrotic over apoptotic cell death.

Effect of Aging on Hydrogen Peroxide-Induced Necrosis. In contrast to apoptosis, cell death by necrosis is associated with a loss of membrane integrity. Taking advantage of a commonly used procedure, we detected necrotic cell death by measuring the release of lactate dehydrogenase (LDH) into cell culture media. As shown in Fig. 5, the release of LDH increases as a function of the age of the fibroblast donor and is greatly enhanced by the presence of hydrogen peroxide in a dose-dependent manner. Moreover, LDH release in the presence of 30 μM hydrogen

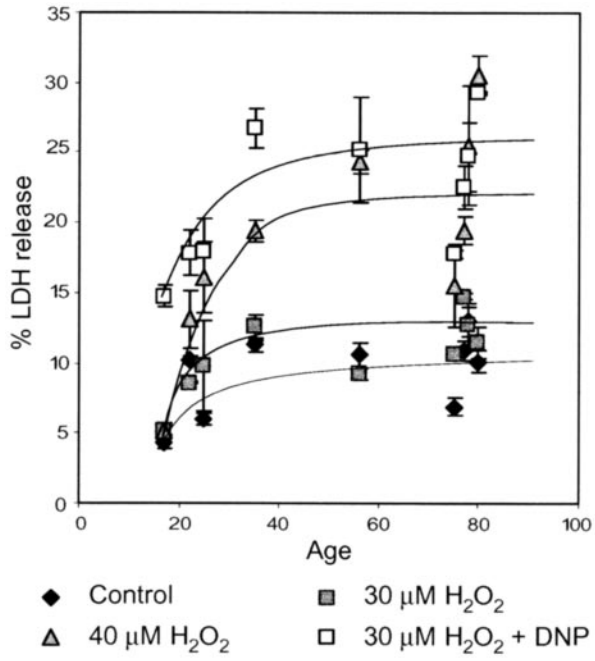

Fig. 5. Hydrogen peroxide-induced necrotic cell death in fibroblasts is age-dependent. Fibroblasts were treated with the indicated concentrations of hydrogen peroxide for 2 h after pretreatment with or without 100 μM DNP for 1 h. Necrotic cell death was monitored by LDH release. Values are the mean of three independent experiments presented with SD.

peroxide was greatly enhanced in samples pretreated with DNP. Similar results were obtained when necrosis was monitored by the propidium iodide intake by using FACS (data not shown).

Effect of Hydrogen Peroxide-Induced Cell Death on Inflammatory Cytokine Release. Necrotic cell death is known to be associated with inflammatory reactions that cause several disorders. Results summarized in Table 1 suggest that cell death of old cells may be associated with a greater inflammatory response than that of young cells, as judged by a greater enhancement in the release of 11 of the 40 cytokines examined. These results may suggest that the old cells, because of their sensitivity to hydrogen

Table 1. Effect of hydrogen peroxide on inflammatory cytokine release in cells from old and young donors

Cells	Fold increase (old/young)
IL-16	1.58*
IL-17	1.47*
IL-6	1.46*
MCP-2	1.37*
sTNF R2	1.33*
IL-12 p40	1.30*
IL-10	1.29*
IL-7	1.29*
sTNF R1	1.24*
IP-10	1.24*
Eotaxin	1.14†
IL-8	0.68*
TNF-α	0.84†

All cells used were treated with 30 μM hydrogen peroxide for 15 h. Values are means of two independent experiments. *, $P < 0.05$; †, $P < 0.1$, statistically different between young (17, 22, 25, and 35 years old) and old (75, 77, 78, and 80 years old) cells (Student's *t* test).

peroxide-induced necrosis, have a relatively higher potential to induce inflammatory reactions than do young cells.

Discussion

Tight linkage between oxidative stress and aging is indicated by the fact that aging is associated with accumulation of oxidized forms of protein (3), nucleic acids (2), and lipids (4), and also by the fact that there is an inverse relationship between the maximum life span of organisms and the age-related accumulation of oxidative damage (3, 15, 19). Protein carbonyls, 8-oxo-2′-deoxyguanosine, acrolein, or 4-hydroxy-2-nonenal, are well established biomarkers of protein, DNA, and lipid oxidation, respectively. The intracellular level of protein carbonyl has become one of the most widely accepted measurements of oxidative stress-dependent cellular damage. In the present study, we observed an age-associated increase in the level of protein carbonyl in mitochondrial fractions as well as in whole-cell lysates and a decrease in the intracellular level of free ATP in human fibroblasts (Figs. 1 and 2). It is interesting to note that the rate of protein carbonyl accumulation is greater in mitochondrial fractions than in whole-cell lysates. This finding is in agreement with the notion that mitochondrial proteins are more susceptible to oxidative modification because the majority of reactive oxygen species are generated in mitochondria. As a consequence, ATP production in the mitochondria would decline with age. Our data also show that there is an age-related decrease in the ability of hydrogen peroxide to induce phosphorylation of c-jun (Fig. 3). Although the role of c-jun phosphorylation in apoptosis is cell type- and cell death stimulus-dependent, c-jun N-terminal kinase-catalyzed phosphorylation of c-jun has been shown to be associated with apoptosis in neuronal cells and primary fibroblasts (20–22). Therefore, the decline in c-jun phosphorylation observed with cells from old donors that have not been treated with hydrogen peroxide may compromise apoptotic signaling in these cells. The complete apoptotic process involves energy-requiring steps, one of which is the formation of the protein complex involving Apaf-1, cytochrome *c*, and procaspase-9 (9, 11). A deficiency of ATP in these steps would prevent downstream processes, including caspase-3 activation. Consistent with results of previous studies showing that low intracellular levels of free ATP might permit apoptotic stimuli to induce necrosis (8, 23–26), we found that a decrease in the intracellular ATP concentration, induced by inhibition of ATPase, glycolysis, or mitochondrial respiration activities, leads to suppression of caspase-3-like activity (Fig. 4*B*) and enhancement of cell necrosis (Fig. 5).

We also monitored several cytokine mRNAs, including IL-1β, IL-6, IL-8, and TNF-α, using RT-PCR and observed that the basal levels of most cytokines studied were higher in fibroblasts derived from young donors than were those derived from old donors (unpublished data). However, we show here that cell aging is associated with significant increases in the hydrogen peroxide-induced leakage of various cytokines (Table 1) that are known to be implicated in the initiation of several age-related inflammatory disorders and diseases, including myocardial infarction, type 2 diabetes, muscle atrophy, osteoporosis, arthritis, and congestive heart failure (27–37). Therefore, these results suggest leakage of cytokine proteins from the intracellular space of necrotic cells that will affect neighboring live cells.

Results of these studies support the thesis that there is an age-related decline in the ability to maintain tissue homeostasis due to a loss in the capacity to carry out apoptosis of oxidatively modified cells and an increase in the sensitivity of such cells to death by necrosis. Moreover, these age-related changes are likely due, in part, to a decline in the intracellular level of ATP and an increase in inflammatory processes associated with the release of a multiplicity of cytokines.

Materials and Methods

Cell Culture and Treatment. Human fibroblasts (AG06234, AG11747, AG09975, AG04447, AG13151, AG13348, AG07135, AG13333, and AG04383) from healthy donors were obtained from the Coriell Institutes for Medical Research (Camden, NJ). Cells were grown to confluency in Eagle's minimum essential medium supplemented with nonessential amino acids, 26 mM Hepes, 2 mM L-glutamine, and 15% FBS (GIBCO). Cell numbers were counted with NucleoCounter (New Brunswick Scientific, Edison, NJ). The same number of cells were plated and grown at 37°C and 5% CO_2/air. Treatment with hydrogen peroxide was carried out in the presence or absence of glucose in serum-free media to avoid the possibility that serum components might differentially affect some of the assays used in this study.

Determination of Carbonyl Protein. Harvested cells and the mitochondria fraction, which was fractionated by using a Mitochondria/Cytosol fractionation kit (BioVision, Mountain View, CA), were lysed in buffer containing 8 M urea. Protein concentrations in each fraction were measured by using the BCA protein assay (Pierce). Twenty micrograms of protein in lysis buffer containing 6% SDS was incubated with 2,4-dinitrophenylhydrazine (5 mM final concentration) for 15 min at room temperature. After the reaction, trichloroacetic acid (10% final concentration) was added to the protein/2,4-dinitrophenylhydrazine mixture. The precipitated protein was collected by centrifugation at 1,650 $\times$ *g* for 3 min and washed three times with acetone to remove free reagent. The final precipitated protein was redissolved in 8 M urea. The samples were further diluted in water to give a 0.2 M final concentration of urea and 250 μg/ml of protein. The same volume from each sample was dotted on nitrocellulose membrane. The membrane was incubated for 1 h at room temperature with 1/150-fold-diluted primary antibody specific to 2,4-dinitrophenyl moiety of the proteins. This step was followed by incubation for 1 h at room temperature with 1/5,000-fold-diluted IRDye 800CW-conjugated affinity-purified anti-rabbit IgG (Rockland, Gilbertsville, PA) against the primary antibody. The 2,4-dinitrophenyl-derivatized proteins were visualized and quantified by using an Odyssey Imaging System (Li-Cor, Lincoln, NE).

Measurement of Intracellular ATP Level. Intracellular ATP was measured by using the ATP Bioluminescence Assay kit HS II (Roche Applied Science). Briefly, cells were harvested, counted, and then lysed with lysis buffer. Fifty microliters from each diluted sample or standard was transferred into a disposable cuvette, and then 50 μl of luciferase reagent was added to it. After mixing, the light emitted was measured and integrated for 10 s by using a TD-20/20 Turner Designs luminometer. The blank value (no ATP) was subtracted from each sample's raw data, and finally ATP concentrations were calculated from the linear part of a standard curve and expressed as fmol per cell.

Immunoblot Analysis. Hydrogen peroxide-treated and untreated cells were harvested and lysed with RIPA buffer containing a phosphatase inhibitor mixture. The same amount of protein in each sample was separated by Nu-PAGE 4–12% Bis Tris gels and transblotted onto a nitrocellulose membrane followed by incubation with blocking buffer. After this step, the membranes were washed and incubated with 1/1,000-fold-diluted phospho-c-jun (Ser-73), c-jun (Cell Signaling Technology, Beverly, MA), or actin (Santa Cruz Biotechnology) antibodies. After washing, the blots were further incubated for 1 h at room temperature with 1/5,000-fold-diluted Alexa Fluor 680 goat anti-mouse IgG (Invitrogen) or IRDye 800CW-conjugated

affinity-purified anti-rabbit IgG. The blots were then washed three times before visualization and quantification with the Odyssey Imaging System.

Cell Death Assay. Fibroblasts from healthy humans of different ages were seeded (0.4×10^4 cells per well) on a 96-well plate. After incubation overnight, the cells were pretreated with or without 2 μM OLG or 100 μM DNP in serum-free medium or 200 μM 2DG in serum and glucose-free medium for 1 h. After washing out the pretreatment medium, cells were treated with hydrogen peroxide in serum-free medium at 37°C for 16 h for the cell viability and apoptotic assays or for 2 h for the LDH release assay. For quantitative analysis of cell viability, 10 μl of a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) solution was added to each well, and, after incubation at 37°C for 2 h in a humidified CO_2 incubator, absorbance at 540 nm was monitored with a microplate reader (SpectraMax Plus 384; Molecular Devices). The values obtained were normalized to those of control cells incubated with vehicle only. The measurement of the caspase-3-like activity was done by using an Apo-ONE homogeneous caspase-3/7 assay (Promega). To measure necrotic cell death, an LDH release assay was carried out by using a CytoTox-ONE Homogeneous Membrane Integrity Assay kit (Promega). Briefly, for quantitative analysis, 100 μl of a reagent was added to each well, and the fluorescence intensity was measured at 560-nm excitation and 590-nm emission after incubation for 2 h in the dark at room temperature. The obtained values were normalized to those of the maximum LDH released (completely lysed) control.

Cytokine Array. Cell cultures were treated with 30 μM hydrogen peroxide in serum-free medium. After incubation for 15 h, supernatants were analyzed for cytokines by using the Human Inflammation Antibody Array III (Raybiotech, Norcross, GA) according to the manufacturer's protocol. Briefly, arrayed antibody membranes were incubated with blocking buffer for 30 min at room temperature. Membranes were then probed with 3 ml of conditioned media for 2 h at room temperature. After washing three times with 2 ml of washing buffer, the membranes were incubated with biotin-conjugated anticytokine antibody diluted in blocking buffer for 2 h at room temperature. Membranes were again washed three times before the addition of 2 ml of 1/2,000-fold-diluted IRDye 800CW-conjugated streptavidin (Rockland, Gilbertsville, PA) for 2 h. Membranes were washed, and the signal was detected and quantified with the Odyssey Imaging System.

This research was supported by the Intramural Research Program of the National Heart, Lung, and Blood Institute, National Institutes of Health.

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