Effect of Hypoxia on Susceptibility of RGC-5 Cells to Nitric Oxide

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PURPOSE. To determine whether retinal neurons become more susceptible to injury by nitric oxide (NO) under hypoxic conditions.

METHODS. Cells from the RGC-5 line were exposed to different concentrations (0.1–100 μM) of S-nitroso-N-acetyl-penicillamine (SNAP), an NO donor, under normoxic and hypoxic (1.0% O2) conditions with 5.5 mM glucose or with no glucose. In some experiments, carboxy-PTIO, a scavenger of NO, was added with SNAP. The SNAP-induced cell injury was determined by the WST-8 assay and by the assessment of phosphatidylserine externalization and changes in hypodiploid DNAs. Alterations of mitochondrial membrane potential, superoxide anion formation, cellular adenosine triphosphate (ATP) contents, and caspase activity were also determined after exposure to SNAP.

RESULTS. Exposure of RGC-5 cells to SNAP (100 μM) significantly decreased the number of living cells cultured under hypoxic conditions with or without glucose. Coadministration of carboxy-PTIO (1.0 μM) suppressed SNAP-induced cell death. SNAP-induced cell death of cells cultured under hypoxia with glucose was accompanied by increased expression of phosphatidylserine and hypodiploid DNAs. These findings indicated that death was mediated in part by apoptosis. In addition, loss of mitochondrial membrane potential, increase of superoxide formation, and activation of caspase was observed. Cyclosporine A, TEMPO, and Z-VAD-FMK suppressed cell death. On the other hand, SNAP depleted the ATP contents of cells cultured under hypoxia without glucose, causing mainly necrotic cell death.

CONCLUSIONS. These results indicate that RGC-5 cells become susceptible to SNAP under hypoxic conditions in which NO may have greater impact on mitochondrial function. (Invest Ophthalmol Vis Sci. 2010;51:2575–2586) DOI:10.1167/iovs.09-4303

Nitric oxide (NO) is a short-lived signaling molecule that was originally identified as an endothelium-derived vasodilator.1,2 NO is synthesized endogenously in the presence of nitric oxide synthase (NOS) by vascular endothelial cells and retinal neurons,3,4 and it regulates ocular blood flow5 and neurotransmission in the retina.6 These physiological roles are mediated through cyclic guanosine monophosphate (cGMP) formation by soluble guanylate cyclase.7

Because NO has a high affinity for heme proteins, another important target of NO is cytochrome oxidase, a mitochondrial enzyme of the electron transport chain.8 Mitochondria, the main cellular energy source, are also involved in nitric oxide signaling and have their own NOS (mtNOS).9,10 NO increases the biogenesis of mitochondria under some conditions, which leads to an increase in the synthesis of adenosine triphosphate (ATP).11 On the other hand, it has been shown that NO inhibits cytochrome oxidase at low oxygen levels and suppresses cellular respiration and oxygen consumption.12,13 It has also been demonstrated that endogenous NO regulates the formation of superoxide anions by modifying the redox state of cytochrome oxidase. This formation of superoxide anions is increased in hypoxia,14 causing damaging effects through the formation of peroxyxynitrite.15

Several studies have demonstrated that mitochondrial dysfunction is commonly observed during excitotoxicity of neurons in the central nervous system.16,17 These studies showed that there was a loss of the mitochondrial membrane potential,16 an increase in the formation of mitochondrial permeability transition pores,17 and an increase in reactive oxygen species.18 Because NOS inhibition can rescue neurons from excitotoxicity19 and exogenous NO causes mitochondrial dysfunction and neuronal death,20 NO has been strongly implicated in neuronal death.

Recently, Uehara et al.21 reported that NO was also associated with neuronal cell death by S-nitrosylation of protein-disulfide isomerase, causing endoplasmic reticulum (ER) stress and neuronal dysfunction. Thus, in addition to the effects on mitochondrial function, NO may induce ER stress and cause the death of retinal neurons.

NO can be neuroprotective and neurodestructive, depending on the redox state22 and on its concentration.23,24 The deleterious effects of NO have been demonstrated under hypoxic conditions, and NOS inhibition protects retinal ganglion cells (RGCs) from retinal ischemia.25,26 However, the effects of NO on retinal ischemia are still contradictory, and neuroprotective effects of NO have also been demonstrated.27–30 These contradictory results from in vivo studies may be confusing because of the variety of actions of NO on various tissues, including retinal neurons and glial cells, and on retinal blood flow.

We hypothesized that retinal neurons become more vulnerable to exogenously applied NO under hypoxic conditions. This is relevant for the optimal treatment of retinal ischemia. To test our hypothesis, we cultured cells from the RGC-5 line under normoxic and hypoxic conditions. In addition, RGC-5 cells were cultured with 5.5 mM glucose (physiological level) or without glucose. Glucose-free medium was used to examine the glycolytic properties of the cells. We examined the effects...
of SNAP on cell death, mitochondrial membrane potential, and superoxide formation under these conditions and determined the involvement of caspase and ER-stress in SNAP-induced cell death.

MATERIALS AND METHODS

Materials

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

RGC-5 Line

The RGC-5 line is a transformed cell line for RGCs that was generously provided by Neeraj Agarwal (University of North Texas Health Science Center, Fort Worth, TX). Cultures of RGC-5 were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Carlsbad, CA) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The cells were passaged by trypsinization every 3 days because of their doubling rate of approximately 24 hours.31

For the experiments, the RGC-5 cells were plated in 10-cm culture dishes or 96-well culture plates in DMEM at a density of 1 x 10⁴ cells/ml then were cultured for 48 hours before use.

Experimental Groups

RGC-5 cells were exposed to 0.1, 1.0, 10, and 100 μM SNAP (Tocris Bioscience, Ellissville, MO), an NO donor, for 16 to 20 hours with different concentrations of oxygen and glucose. In some experiments, carboxy-PTIO (Dojindo, Kumamoto, Japan), a scavenger of NO, was added to the medium with SNAP. Before exposure to SNAP, the RGC-5 cells were washed three times in PBS, and the medium was switched to the medium with SNAP. Before exposure to SNAP, the RGC-5 cells were cultured in 10-cm dishes and in an anaerobic chamber (BioSpherix, Redfield, NY) for 20 minutes before use. Culture dishes were placed in a CO₂ incubator (95% air with 5.0% CO₂) for normoxic condition and in an anaerobic chamber (BioSpherix, Redfield, NY) for hypoxic condition (1% O₂, 94% N₂, 5% CO₂). SNAP exposure was performed concurrently when culture media were changed to the four different conditions. Thus, cells were exposed to SNAP for 16 to 20 hours under normoxic or hypoxic conditions with or without glucose.

WST-8 Assay

The relative number of living cells was determined with a WST-8 assay kit (CCK-8; Dojindo). WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt produces a water-soluble formazan dye on bioreduction in the presence of an electron carrier, 1-methoxy PMS. After exposure to SNAP under the different conditions, the medium was replaced by a 10% WST-8 solution in DMEM (100 μL). The number of living cells in each well was determined by measuring the optical density (OD) at 460 nm with a microplate reader (SH-100 Laboratory; Corona, Ibaraki, Japan) and was expressed as a value relative to the control value.

Flow Cytometry Analyses

We also evaluated SNAP-induced cell injury by assaying for the expression of the phosphatidylserine and hypodiploid DNAs (FACSAria; Becton Dickinson, San Jose, CA). Changes in mitochondrial membrane potential were also determined. In each assay, 1 x 10⁴ cells were analyzed by acquisition and analysis software (Cell Quest; Becton Dickinson) to acquire and quantify the fluorescence signal intensities and to make dot-density plots.

After exposure to SNAP, the RGC-5 cells cultured in 10-cm dishes were harvested by trypsinization. The cells were centrifuged at 2000 rpm for 5 minutes, and the pellet was resuspended in assay buffer (1.0 mL) at a density of 2.0 x 10⁵ cells/ml. Then 5 μL FITC-conjugated annexin V and 10 μL propidium iodide (PI) were added to each sample. Annexin V-FITC- and PI-stained cells were excited by a 488-nm laser light, and they were collected in the FITC (515–545 nm) and PI (600–620 nm) channels according to emission wavelength.

To examine the DNA contents, harvested RGC-5 cells were fixed in 70% ethanol for 30 minutes at 4°C. The cells were then gently spun down, and the ethanol was removed. The permeabilized cells (2.0 x 10⁵) were resuspended in PBS (100 μL) and incubated with RNase A (1.0 μL) for 30 minutes at room temperature. After PBS (850 μL) was added, the cells were stained with PI (50 μg/mL). The percentage of cells with sub-G1 DNA content was taken as a measure of the apoptosis of the cell population.

Mitochondrial membrane potential is generated by the mitochondrial electron transport chain; loss of mitochondrial membrane potential has been shown to be critical for the initiation of apoptosis.52 We assessed changes in mitochondrial membrane potential with the dye (MitoLight; Chemicon, Rosemont, IL). After exposure to SNAP under either normoxic or hypoxic conditions in the presence of glucose (5.5 mM), harvested cells were incubated with 1.0 mL solution (900 μL distilled water, 1 μL dye [MitoLight; Chemicon] and 100 μL 10X incubation buffer) for 15 minutes according to the manufacturer’s instructions. In healthy cells, the accumulation and aggregation of the dye in the mitochondria yields red fluorescence, but the dye remains in the cytoplasm and gives off green fluorescence when mitochondrial membrane potential is depolarized. We thus analyzed the differences in green and red fluorescence intensities using the FITC and PI channels.

Formation of Superoxide and Peroxynitrite

To determine whether SNAP increases the formation of superoxide and peroxynitrite and to determine how the proportion of these compounds is changed by hypoxia, we measured these radicals semiquantitatively with specific fluorogenic probes, hydroethidine53 and dihydrodihydrodiamine-123,54 respectively. After completion of the exposure to SNAP (100 μM) either in normoxia or in hypoxia with 5.5 mM glucose, cells were harvested by trypsinization. Then, either hydroethidine (1 μg/mL; 5.2 μM) or dihydrodihydrodiamine-123 (1.0 μM) was added to the wells for 30 minutes at 37°C in the dark. After gentle washing with PBS, cells were resuspended in assay buffer (1.0 mL) and subjected to FACS analysis. The intensity of the ethidium or rhodamine fluorescence of the cells was determined with a 488-nm excitation laser light and emission of the PI channel for ethidium55 and the FITC channel for rhodamine.56

In addition to the fluorometric analyses, we also measured nitrotyrosine formation, a biological marker for peroxynitrite.56 After the completion of SNAP treatment under normoxic or hypoxic conditions in the presence of glucose (5.5 mM), cells grown in each well of the 96-wells culture plate were lysed with 50 μL lysis buffer (Toyol ink, Tokyo, Japan). The lysed samples (100 μL) or standard solutions (100 μL) of nitrotyrosine were transferred to the wells of NWLSS nitrotyrosine enzyme-linked immunosorbant assay (ELISA) kit (Northwest Life Science, Vancouver, BC, Canada), and measurements were performed according to the manufacturer’s protocol. The amount of nitrotyrosine in each well was determined by measuring OD at 450 nm with a microplate reader (SH-100 Laboratory; Corona) and was normalized by the protein concentrations.

Determination of ATP Content

Cells grown in 96-well culture plate were exposed to SNAP (0.1–100 μM) in different oxygen and glucose concentrations. After completion of the exposure, cells in each well were lysed in 50 μL lysis buffer (Toyol ink), and the lysed sample (50 μL) was transferred to white, solid-bottom, 96-well assay plates (Corning, Lowell, MA).
The amount of ATP was measured by a reaction between sample and luciferin-luciferase assay solution (Toyo Ink) with and without the addition of internal ATP standard solution (10 mM, 10 μL). Luminescence was measured with a lumino illuminator (Luminoskan; Thermo, Vantaa, Finland). ATP levels were normalized by the protein concentrations and expressed as values relative to the control.

**Determination of Caspase Activity**

RGC-5 cells were cultured in black, clear-bottom, 96-well culture plates (Iwaki, Tokyo, Japan) under the different conditions. Some of the cells were exposed to SNAP (100 μM) with or without carboxy-PTIO (1.0 μM). Caspase-3/7 activity was measured with an assay kit (Apo-ONE Homogeneous Caspase-3/7; Promega, Madison, WI) according to the manufacturer’s protocol. Briefly, the assay (Apo-One Homogeneous Caspase-3/7; Promega) reagent (100 μL) was added directly to each well at room temperature. The plates were shaken at 500 rpm for 30 seconds, and caspase-3/7 activation was determined by the fluorescence intensity (excitation 480 nm, emission 520 nm) with a fluorescence plate reader (Fluoscan Ascent FL; Thermo), and intensity was expressed in arbitrary fluorescence units per microgram of protein.

**Determination of Endoplasmic Reticulum Stress by Immunoblotting**

Functional enzymes or structural proteins must be properly folded (i.e., have proper conformation). Most membrane and secretory proteins are properly folded in the ER, but, under stressful conditions, these proteins are often misfolded. The misfolded proteins induce the so-called ER stress response, and ER stress activates well-defined protective and death pathways. We determined the expression of ER stress-associated proteins, glucose-regulated protein 78 (GRP78)/BiP, and C/EBP-homologous protein (CHOP) by immunoblotting. RGC-5 cells, cultured under different conditions with or without the addition of SNAP (100 μM), were lysed using a cell lysis buffer (R0278) with protease (P8340), phosphatase inhibitor cocktails (P2850 and P5726;
Sigma-Aldrich), and 1 mM EDTA. Cell lysates were solubilized in SDS sample buffer, separated on 10% SDS-polyacrylamide gels, and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). The transfers were blocked for 1 hour at room temperature (5% Blocking One-P; Nakarai Tesque, Kyoto, Japan) in 10 mM Tris-buffered saline with 0.05% Tween 20 (TBS-T) and incubated overnight at 4°C with the primary antibody. The transfers were then rinsed with TBS-T and incubated for 1 hour at room temperature in horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (Pierce, Rockford, IL) diluted 1:2000. The immunoblots were developed using chemiluminescence (Super Signal West Femto Maximum Sensitivity Substrate; Pierce) and were examined with a digital imaging system (FAS-1000; Toyobo, Osaka, Japan). The primary antibodies used were mouse anti-BiP (BD Bioscience, San Jose, CA), mouse anti-CHOP (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-actin (Santa Cruz Biotechnology).

**Protein Determination**

RGC-5 cells were cultured in 96-well culture plates (Iwaki, Tokyo, Japan) under the different conditions. After completion of the treatment, cells were lysed with lysis buffer (Toyo Ink), and the protein concentration was determined (BCA Protein Assay Kit; Pierce).

**Statistical Analyses**

Data are expressed as mean ± SEM unless otherwise noted. Statistical analysis was conducted with one-way analysis of variance (ANOVA); if a significant change was detected, a post hoc test was performed for statistical comparisons among groups. The Dunnett test was used to assess differences from the control, and the Scheffe test was performed to test significance between two arbitrary groups. Student’s t-tests were used to compare the differences between two groups. The level of significance was set at P < 0.05.
RESULTS

Dose-Dependent Effects of SNAP on Death of RGC-5 Cells

The effects of different concentrations of SNAP on the death of RGC-5 cells cultured under different conditions and determined by WST-8 assays are shown in Figure 1. Data are expressed as values relative to the control (100%), which were the untreated cells cultured under normoxia with 5.5 mM glucose. In the presence of glucose, SNAP (0.1–100 μM) did not cause cell death when the cells were cultured under normoxia (Fig. 1A), whereas 100 μM SNAP caused a significant (P < 0.05; Dunnett) decrease in the relative number of living cells cultured under hypoxia (Fig. 1B). Glucose deprivation alone reduced the percentage of living cells to 55.2% ± 2.5% and 51.2% ± 2.1% of the control cultured in normoxia and hypoxia, respectively (Figs. 1C, 1D). However, the differences in these levels were not significant (P > 0.27, t-test). Under this glucose-deprived condition, SNAP (0.1–100 μM) did not cause a further increase of dead cells when cultured in normoxia (Fig. 1C).

In contrast, SNAP (100 μM) caused a significant (P < 0.05, Dunnett) reduction in the relative number of living cells cultured in hypoxia in the absence of glucose (OGD; Fig. 1D). Thus, further analyses were made to determine the mechanisms of cell death using 100 μM SNAP.

When carboxy-PTIO (1 μM) was added to the medium with SNAP (100 μM), SNAP-induced cell death under hypoxic conditions, either with (Fig. 1E) or without (Fig. 1F) glucose, was significantly suppressed (P < 0.05, Dunnett).

Detection of Annexin-V/PI Stained Cells by Fluorescence-Activated Cell Sorting Analysis

Representative dot plots obtained by FACS of Annexin-V/PI-stained cells after exposure to SNAP (100 μM) under the different conditions are shown in Figure 2. Consistent with the results of the WST-8 assays, SNAP increased the population of cells stained positively by either Annexin-V or PI under hypoxic conditions. SNAP (100 μM) increased the percentage of Annexin-V+/PI- cells in the hypoxic media with glucose (Fig. 2D), whereas it mainly increased the Annexin-V+/PI+ cells cultured in the OGD media (Fig. 2H). In normoxia, SNAP (100 μM) did not significantly increase Annexin-V or PI- cells in the presence of glucose (Fig. 2B), whereas it decreased Annexin-V+/PI- cells but increased Annexin-V-/PI+ cells in the absence of glucose (Fig. 2F).

Results of the quantitative analyses, including the effects of lower concentrations of SNAP, are shown in Figure 3. In the normoxic media with 5.5 mM glucose, SNAP (0.1–100 μM) did not increase the number of Annexin-V or PI- cells from control levels (Fig. 3A).

In the hypoxic media with 5.5 mM glucose, SNAP increased (P = 0.001) the percentages of cells stained positively by Annexin-V and negatively by PI (representative staining pattern of apoptosis) and decreased (P = 0.02) the number of living cells stained negatively by these dyes in a dose-dependent manner (ANOVA). More specifically, SNAP at 10 and 100 μM significantly (P < 0.05, Dunnett) increased the percentages of Annexin-V+/PI- cells from 4.7% ± 0.6% to 11.2% ± 1.8% and

Asterisks: significant (P < 0.05, Dunnett) differences from their counterpart controls (without the addition of SNAP). Q1, cells stained positively with annexin-V and negatively stained with PI; Q2, cells stained positively with both annexin-V and PI; Q3, cells stained negatively with these dyes; Q4, cells stained negatively with annexin-V and positively with PI.
In the normoxia and glucose-deprived media, the effects of SNAP were variable, depending on its concentrations. For example, SNAP at 100 μM significantly \( (P < 0.05,\) Dunnett) decreased the percentages of Annexin-V/PI\(^{-}\) cells from 6.7% ± 1.0% to 2.8% ± 0.1%, whereas it significantly \( (P < 0.05,\) Dunnett) increased the percentage of cells with necrotic staining patterns (i.e., Annexin-V/PI\(^{-}\) cells from 4.4% ± 1.4% to 11.6% ± 0.7%; Fig. 3C). Lower concentration of SNAP (0.1 and 1.0 μM) significantly \( (P < 0.05,\) Dunnett) increased the percentage of Annexin-V/PI\(^{-}\) cells from 6.7% ± 1.0% to 10.6% ± 1.1% and 10.9% ± 0.4%, respectively, indicating an increase in the number of deaths from apoptosis (Fig. 3C). SNAP at 10 μM caused a significant \( (P < 0.05,\) Dunnett) reduction in the percentage of Annexin-V/PI\(^{-}\) cells from 21.2% ± 1.5% to 14.0% ± 0.8% without affecting the population of the other cell types (Fig. 3C).

In the OGD media, SNAP at 100 μM caused a significant \( (P < 0.05,\) Dunnett) increase in the number of Annexin-V/PI\(^{-}\) cells from 17.3% ± 3.1% to 72.7% ± 2.2%, with a significant reduction of Annexin-V/PI\(^{-}\) cells (Fig. 3D). Lower concentrations of SNAP (0.1–10 μM) had no significant effects.

**Detection of Hypodiploid DNA by FACS**

Representative histograms of the DNA concentrations after PI staining and FACS are shown in Figures 4A through 4H, and the quantitative analyses are shown in Figure 4I. In the presence of glucose, SNAP did not increase the population of sub-G1 fraction under normoxia (Fig. 4B). In contrast, SNAP (100 μM) significantly increased the percentage of sub-G1 fraction under hypoxia (Fig. 4D). SNAP (100 μM) again did not increase the population of apoptotic cells under normoxia (Fig. 4F). Although a significant increase \( (P < 0.05 vs. control;\) Fig. 4I) in apoptotic DNA fragmentation was observed when cells were cultured in the OGD media (Fig. 4G), the addition of SNAP (100 μM) to the OGD media did not cause further increases of the hypodiploid DNA (Figs. 4H, 4I).

Thus, on the basis of Annexin-V/PI staining and DNA content, SNAP (100 μM) caused, at least in part, the apoptotic death of RGC-5 cells under hypoxia in the presence of glucose. On the other hand, because the SNAP-induced increase in the Annexin-V/PI\(^{-}\) cells in the OGD media (by 2.7 times) was not accompanied by an increase in the percentage of hypodiploid DNA, cell death was more likely caused by necrosis.

**Intracellular ATP Contents**

ATP levels of cells cultured in the presence or absence of SNAP (0.1–100 μM) relative to the values of the control are shown in Figure 5. SNAP (0.1–100 μM) exposure did not decrease the ATP levels significantly in cells cultured in normoxic conditions (Fig. 5A) but rather increased the ATP levels in the absence of glucose (Fig. 5C). In contrast, SNAP decreased the intracellular ATP contents when cells were cultured in hypoxia (Figs. 5B, 5D).

In the presence of glucose, ATP levels of cells cultured in hypoxia were still as high as 80.0% ± 3.4% of controls (Fig. 5B). The addition of SNAP (100 μM) to the media decreased ATP levels significantly \( (P < 0.05,\) Dunnett), to 56.6% ± 3.5% of control (Fig. 5B). At lower concentrations of SNAP (0.1–10 μM), there was also a significant decrease in ATP levels \( (P < 0.05,\) Dunnett) from those without added SNAP (Fig. 5B). When the glycolytic system was restricted by glucose deprivation, the ATP level of cells cultured in normoxia (36.2% ± 2.3%) was not significantly different \( (P = 0.60,\) t-test) from that

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**FIGURE 4.** Representative histograms of the DNA level obtained by FACS after PI staining (A–H). Left: counterpart controls (without the addition of SNAP) of the (right) cells exposed to SNAP (100 μM). Culture conditions are indicated at the left. Vertical and horizontal axes represent the number of cells and the PI signal intensity (log units), respectively. (I) Quantitative analyses of sub-G1 fraction population \((n = 4–6\) for each condition). SNAP significantly increased the fraction in hypoxia and in the presence of glucose. SNAP also decreased the percentage of the fraction in the cells cultured in hypoxia in the absence of glucose (OGD).

16.5% ± 2.1%, respectively, whereas SNAP at these concentrations also significantly \( (P < 0.05,\) Dunnett) decreased the percentages of Annexin-V/PI\(^{-}\) cells from 87.3% ± 1.6% to 77.7% ± 2.0% and 68.3% ± 4.0%, respectively (Fig. 3B). SNAP at lower concentrations (0.1 and 1.0 μM) did not alter the percentages of either Annexin-V\(^{-}\) or PI\(^{-}\) cells.
In contrast, SNAP at a dose of 10 μM increased the levels in cells cultured in normoxia significantly (P < 0.05, Dunnett; C).

Changes in Mitochondrial Membrane Potentials

Changes in the mitochondrial membrane potentials by SNAP (100 μM) were determined on cells cultured under normoxia or hypoxia in the presence of glucose. Representative histograms of the mean mitochondrial membrane potentials determined by staining the cells with dye (MitoLight; Chemicon) and analyzed by FACS are shown in Figures 6A and 6B.

Mitochondrial membrane potentials in cells exposed to SNAP (100 μM) under hypoxia had two peaks with different green fluorescence intensities, whereas the control cells, which were cultured in normoxia in the presence of glucose, had a single peak of lower intensity (Fig. 6A). On the other hand, the mean red fluorescence intensity of PI in cells exposed to SNAP in hypoxia was lower than that in the control cells (Fig. 6B). These changes showed a decrease in the ratio of the mean intensities of PI/FITC of cells exposed to SNAP in hypoxia, indicating depolarization of mitochondrial membrane potential.

Ratios obtained from cells exposed to SNAP (0.1–100 μM) under either normoxia or hypoxia in the presence of glucose are shown in Figures 6C and 6D. Under normoxic conditions and in the presence of glucose, SNAP (0.1–100 μM) did not decrease mitochondrial membrane potential significantly (P > 0.05, Dunnett; Fig. 6C). In contrast, SNAP at doses of 10 and 100 μM caused a significant (P < 0.05, Dunnett) reduction of the ratios under hypoxia in the presence of glucose, whereas lower concentrations of SNAP (0.1 and 1.0 μM) did not change the ratios significantly (Fig. 6D).

Formation of Superoxide and Peroxynitrite

Changes in the formation of superoxide and peroxynitrite caused by SNAP (100 μM) were determined in cells cultured either in normoxia or hypoxia in the presence of glucose. Representative histograms of the intensity of the intracellular ethidium and rhodamine fluorescence obtained by FACS are shown in Figures 6E and 6G, respectively. SNAP did not alter the formations of superoxide anion (Fig. 6E, upper panel) and peroxynitrite (Fig. 6G, upper panel) under normoxia in the presence of glucose but did cause a rightward shift of these histograms, indicating an enhancement of these fluorescence intensities under hypoxia in the presence of glucose (Figs. 6E, 6G; lower panels).

Mean ethidium and rhodamine fluorescence intensities of cells exposed to SNAP (100 μM) are shown in Figures 6F and 6H, respectively. SNAP significantly (P = 0.02, Scheffé; Fig. 6F) increased the ethidium fluorescence intensity of cells cultured under hypoxia with glucose. However, SNAP did not change intensity significantly under normoxia in the presence of glucose.

Similarly, SNAP significantly (P = 0.01, Scheffé; Fig. 6H) increased the rhodamine fluorescence intensity of cells cultured under hypoxia with glucose but did not increase the intensity under normoxia. Hypoxia alone did not enhance these fluorescence intensities in RGC-5 cells (Figs. 6F, 6H).

The nitrotyrosine level of control cells assessed by ELISA was 0.6 ± 0.1 nmol/μg protein (n = 5), and that in cells exposed to SNAP (100 μM) under normoxia with glucose was 0.8 ± 0.2 nmol/μg protein (n = 5). The nitrotyrosine level of cells exposed to SNAP (100 μM) in hypoxic media with glucose was 2.3 ± 0.7 nmol/μg protein (n = 5), which was significantly (P < 0.05, Dunnett) higher than control the level. The nitrotyrosine level in cells cultured in hypoxia without the
FIGURE 6. SNAP-induced changes in mitochondrial membrane potentials (A–D) and formation of superoxides (E–H). (A, B) Representative histograms of FITC (A) and PI (B) fluorescence intensity stained by dye. Vertical and horizontal axes represent number of cells and fluorescence intensity, respectively. SNAP formed two peaks of green (FITC) fluorescence. The presence of the peak with higher intensity increased the mean fluorescence intensity (A), whereas SNAP decreased mean red (PI) fluorescence intensity (B). (C, D) Mitochondrial membrane potentials are represented by ratios of red (PI)/green (FITC) mean fluorescence intensities. SNAP (0.1–100 μM) did not decrease the membrane potentials indicated by the ratios under normoxia with glucose (C). In contrast, SNAP (10 and 100 μM) decreased the ratios significantly (P < 0.05, Dunnett), causing depolarization of membrane potentials under hypoxia with glucose (D). (E, G) Representative histograms of ethidium (E) and rhodamine (G) fluorescence intensities of cells cultured in normoxia (upper) and hypoxia (lower) in the presence of 5.5 mM glucose. Vertical and horizontal axes represent number of cells and fluorescence intensity, respectively. SNAP caused a rightward shift of the fluorescence intensities in hypoxia (E, G, lower). (F, H) Ethidium (F) and rhodamine (H) fluorescence intensities (mean ± SEM) are shown for each condition. SNAP significantly increases the ethidium (P = 0.02, Scheffé; F) and rhodamine intensities (P = 0.01, Scheffé; H) in hypoxia in the presence of glucose but not in normoxia. Hypoxia alone did not alter these fluorescence intensities.
addition of SNAP was 1.3 \pm 0.1 \text{nmol/\mu g protein} (n = 5) and was not significantly different from the control level.

**Activation of Caspase**

To determine whether the SNAP-induced cell injury was mediated by activating caspase, caspase activity was assessed with an assay kit (Apo-ONE Homogeneous Caspase-3/7; Promega) in the presence or absence of carboxy-PTIO (1.0 \text{M}); results are presented in Figure 7A. Exposure to SNAP (100 \text{M}) induced a significant (P = 0.001, Scheffe) increase in caspase activity, by 59.1%, in cells cultured under hypoxia in normoxia. Caspase activity in cells cultured in hypoxia in the absence of glucose (OGD) was also significantly higher (P < 0.05, Dunnett) than in control cells. Addition of carboxy-PTIO suppressed these activations. In addition, SNAP decreased (P = 0.02, Scheffe) the caspase activity of cells cultured in normoxia in the presence of glucose. Under normoxic conditions, SNAP (100 \text{M}) significantly (P = 0.02, Scheffe) decreased caspase activity, indicating some neuroprotective role of NO under this condition (Fig. 7A).

**Effects of Cyclosporine A, TEMPOL, and Inhibitors of Caspase on SNAP-Induced Cellular Injury**

To determine whether the opening of mitochondrial pores secondary to the loss of mitochondrial membrane potential was involved in SNAP-induced cell injury, the effects of cyclosporine A on cell viability were determined with the WST-8 assay. RGC-5 cells were exposed to SNAP (100 \text{M}) under hypoxia with glucose, and cyclosporine A and TEMPOL significantly (P < 0.05, Dunnett) suppressed the reduction in the number of living cells caused by SNAP (100 \text{M}) seen in hypoxia in the presence of 5.5 mM glucose. Thus, the caspase-mediated cell death would occur by glucose deprivation under normoxia. The addition of SNAP (100 \text{M}) significantly (P = 0.02, Scheffe) decreased caspase activity, indicating some neuroprotective role of NO under this condition (Fig. 7A).
SNAP-induced cell injury under hypoxia in the presence of glucose. TEMPO\(\text{L}\) (100 nM) and Z-VAD-FMK (10 \(\mu\)M) partially protected RGC-5 cells against SNAP-mediated cell death (Fig. 7C).

**Determination of Involvement of Endoplasmic Reticulum Stress**

Representative immunoblots showing changes in the protein levels of GRP78/BiP and CHOP of cells cultured under different conditions, with and without the addition of SNAP (100 \(\mu\)M), are shown in Figure 8A. The changes are standardized to the actin levels and quantified in Figures 8B and 8C.

Under all culture conditions, the addition of SNAP (100 \(\mu\)M) tended to increase GRP78/BiP protein expression, and a significant increase from the control level was seen in cells cultured in normoxia without glucose. However, under other conditions, the SNAP-induced increase of GRP78/BiP proteins was not significant from their counterparts without the addition of SNAP or from the controls. Similarly, the expression of CHOP was significantly increased compared to control (Dunnett) elevated in cells cultured in normoxic glucose-deprived media either with or without addition of SNAP (100 \(\mu\)M). However, the expression of CHOP was very weak compared with that of GRP78/BiP, and the addition of SNAP did not change the expression of CHOP of cells in the other culture conditions.

**DISCUSSION**

Our results showed that RGC-5 cells became susceptible to SNAP under hypoxic conditions, but SNAP can also be neuroprotective under normoxia under some conditions. The SNAP-induced cell death under hypoxia in the presence of glucose occurred primarily by apoptosis and by necrosis in the absence of glucose. The apoptotic cell death caused by SNAP under hypoxia in the presence of glucose was accompanied by caspase activation and formation of superoxide anion and peroxynitrite, whereas hypoxia itself did not affect the redox state of the cells. Because carboxy-PTIO protected RGC-5 cells against SNAP-induced toxicity and suppressed the activation of caspase, the action of SNAP was most likely mediated by NO.

The origin of the RGC-5 line has been recently recharacterized. The RGC-5 line, designated as a transformed rat retinal ganglion cell line, is actually of mouse origin. However, when differentiated by exposure to staurosporine, the RGC-5 cells express some proteins characteristic of RGCs, including Thy1 and NMDA receptor, which indicates that RGC-5 is a precursor cell line for retinal ganglion cells. Because the aim of our study was to determine whether the damaging effects of NO on retinal neurons were augmented under hypoxia, the origin of the RGC-5 cell line was less relevant to our results.

It has been shown that apoptosis and necrosis may share death signaling pathways, and their presence is determined by the cellular ATP levels. RGC-5 cells probably have very potent glycolytic activity because the ATP levels were as high as 80.0% ± 3.4% of the control when cells were cultured under hypoxia in the presence of glucose. SNAP (100 \(\mu\)M) decreased cellular ATP levels to 56.6% ± 3.5% of the control under hypoxia in the presence of glucose, but the levels were still high enough to induce apoptotic death of the RGC-5 cells.

The effects of SNAP (100 \(\mu\)M) on cell death became more significant when the glycolytic properties were limited by glucose deprivation under hypoxia (OGD). We suggest that the addition of SNAP (100 \(\mu\)M) to the OGD media could have arrested mitochondria respiration and almost completely depleted the ATP levels leading to necrosis. Consistently, the SNAP-induced increase of caspase activity remained at 40.0% under this condition, which was relatively small compared with the increase of annexin V and PI^+^ cells, which was by 2.7 times.

In contrast, we found some protective effects of SNAP (10 and 100 \(\mu\)M) with an increase of cellular ATP levels (Fig. 5C) and a suppression of caspase activities (Fig. 7A) when it was added to the normoxic glucose-deprived media. We have not determined the mechanisms for these neuroprotective effects.
However, NO can increase the biogenesis of mitochondria and inhibit apoptosis by nitrosylation of the caspases. NO can also interact with ATP-sensitive potassium channels. These effects of NO may account for the neuroprotective properties of SNAP under normoxia in the absence of glucose.

The SNAP-induced apoptotic death of RGC-5 cells, observed in hypoxia in the presence of glucose, was partially mediated by oxidative stress because reactive oxygen species formation was increased, and TEMPO was able to reduce the number of dead cells. Although hypoxia may increase the formation of superoxides under some conditions, hypoxia alone did not increase the formation of superoxides in our preparations. Thus, it should be emphasized that exogenously applied NO can act on the mitochondria, the main intracellular source of superoxides, and can change the redox state, increasing the formation of superoxides under hypoxic conditions. An increase of superoxides might cause a formation of permeability transition pores, which may lead to cytochrome c release into the cytoplasm. Because cyclosporine A and Z-VAD-FMK rescued the RGC-5 cells from SNAP-induced death, our results support the idea that NO causes apoptotic cell death through the formation of mitochondrial permeability transition pores and caspase activation.

The expressions of GRP78/BiP and CHOP in cells exposed to SNAP were inconsistent under some conditions, possibly because these two proteins have different properties. GRP78/BiP protein acts as an ER resident molecular chaperon that is induced by ER stress and refolds the unfolded proteins and thus maintains the homeostasis of the ER and could be neuroprotective. We found that SNAP tended to increase GRP78/BiP expression under all culture conditions. On the other hand, CHOP is a member of the C/EBP homology region protein family that is induced by ER stress and is more closely related to ER-mediated apoptosis. Thus, a significant increase in the expression of CHOP in cells cultured in normoxic glucose-deprived media (Fig. 8C) suggested that ER stress was involved in the death of RGC-5 cells under this condition. Indeed, ER stress can be induced by glucose starvation, though the addition of SNAP (100 μM) to this medium caused a further increase of CHOP that did not decrease the cell viabilities or ATP contents in other assays. In addition, SNAP did not change the expression of CHOP in cells cultured in hypoxia either in the presence or absence of glucose, whereas SNAP caused a significant increase in cell death. Taken together, ER stress might have played a minor role in our results. Clearly, the effects of NO on ER stress should be investigated using other strategies. In addition, whether the effect of cGMP is involved in SNAP-induced cell death remains to be determined.

Our results may well explain the massive loss of RGCs caused by the activation of inducible NO synthase from glial cells that is seen in retinal ischemia. Mitochondrial dysfunction and energy depletion may be the mechanism for this. Because the retina is the most metabolically active tissue, retinal ischemia may occur under various conditions, including central retinal artery occlusion, ischemic retinal vein occlusion, and diabetic retinopathy. In addition, retinal ischemia is one of the mechanisms that have been shown to cause pathologic changes in some types of glaucoma. The death of retinal neurons may occur through necrosis or apoptosis, depending on tissue oxygen and NO levels in these conditions. Because the RGC-5 cells have mitotic abilities and lack several characteristic proteins of mature RGCs, our results are not necessarily relevant to mature RGCs. However, the axons of RGCs require mitochondria as energy suppliers. Although NO is known to control energy metabolism in glial cells by upregulating glycolytic activity, SNAP (0.1–100 μM) significantly decreased cellular ATP contents in RGC-5 cells. Thus, when treating patients with retinal and optic nerve ischemia by medication with NO-donating action, the adverse effects of NO under hypoxic conditions should be considered.

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