

Epigallocatechin Gallate Protects U937 Cells Against Nitric Oxide-Induced Cell Cycle Arrest and Apoptosis

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Abstract Ingesting phenolic phytochemicals in many plant products may promote health, but the effects of phenolic phytochemicals at the cellular level have not been fully examined. Thus, it was determined if the tea phenolic phytochemical, epigallocatechin gallate (EGCG), protects U937 human pro-monocytic cells against the nitrogen free radical, nitric oxide (\bullet NO). Cells were incubated for 4–6 h with 500 μ M S-nitrosoglutathione (GSNO), which generates \bullet NO, but this did not induce single-strand breaks in DNA. Nevertheless, $82 \pm 4\%$ of GSNO-treated cells, compared to only $39 \pm 1\%$ of untreated cells, were arrested in the G_1 -phase of the cell cycle. However, dosing the GSNO-treated cells with 9, 14, or 18 μ g/ml of EGCG resulted in only $74 \pm 8\%$, $66 \pm 1\%$, and $43 \pm 3\%$ of the cells, respectively, in the G_1 -phase. Exposing cells to GSNO also resulted in the emergence of a sub- G_1 apoptotic cell population numbering $14 \pm 3\%$, but only $5 \pm 2\%$, $5 \pm 1\%$, and $2 \pm 0\%$ upon dosing of the GSNO-treated cells with 9, 14, and 18 μ g/ml of EGCG, respectively. Furthermore, exposing cells to GSNO resulted in greater cell surface binding of annexin V-FITC, but binding was 41–89% lower in GSNO-treated cells dosed with EGCG. Collectively, these data suggest that \bullet NO or downstream products induced cell cycle arrest and apoptosis that was not due to single-strand breaks in DNA, and that EGCG scavenged cytotoxic \bullet NO or downstream products, thus reducing the number of cells in a state of cell cycle arrest or apoptosis. *J. Cell. Biochem.* 81:647–658, 2001. © 2001 Wiley-Liss, Inc.

Key words: apoptosis; cell cycle; epigallocatechin gallate; nitric oxide

Phenolic phytochemicals are a large class of natural substances that can be found in many edible plant products such as fruits, vegetables, herbs, and spices. Because of their diverse chemical structures [Shahidi and Wanasundara, 1992], phenolic phytochemicals have many interesting properties. For example, their antioxidant activity [Rice-Evans et al., 1995] enables them to effectively scavenge free radicals. As previously demonstrated in non-cellular models, phenolic phytochemicals scavenge reactive oxygen species (ROS) such as hydroxyl radical (\bullet OH) [Hanasaki et al., 1994], hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) [Cai et al., 1997], as well as reactive nitrogen

species (RNS) such as nitric oxide (\bullet NO) [van Acker et al., 1995] and peroxynitrite ($ONOO^-$) [Haenen et al., 1997]. Because ROS and RNS may be involved in the development of numerous chronic degenerative diseases [Halliwell and Gutteridge, 1990], it is critical for optimum health to keep ROS and RNS at reasonably low levels by way of antioxidants such as phenolic phytochemicals.

When present in excess, RNS such as \bullet NO can react with DNA [Halliwell, 1999] and other critical cellular components. If structural or covalent modification of DNA has been sustained, cells arrest in the G_1 -phase of the cell cycle in an attempt to repair the damaged DNA. If such DNA lesions are irreparable, cells undergo programmed cell death or apoptosis. On the other hand, under abnormal conditions such as faulty DNA repair [Sancar, 1995] or presence of a defective tumor suppressor gene [Lee et al., 1994], the DNA lesions can give rise to mutations that allow cancer cells to evolve. Because \bullet NO-induced damage to DNA could lead eventually to mutagenesis and cancer

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[Halliwell, 1994], there is substantial interest in examining free radical scavengers or antioxidants that inhibit •NO.

The daily consumption of tea beverage, a food rich in phenolic phytochemicals, is believed to have many beneficial effects on health, especially in preventing mutagenesis and carcinogenesis that are associated with free radicals. Epidemiological studies link tea beverage drinking to a lower incidence of certain types of cancer [Ji et al., 1996; Katiyar and Mukhtar, 1996]. Consumption of tea extract by animals reduced the degree of tumorigenesis when they were exposed to chemical carcinogens [Katiyar and Mukhtar, 1996]. In explaining the putative cancer chemopreventive effects of tea beverage, its constituent phenolic phytochemicals, particularly epigallocatechin gallate (EGCG), have been examined for antioxidant or free radical-scavenging activity and antitumorogenic effects. EGCG scavenged •NO reagent [van Acker et al., 1995], as well as inhibited ionizing radiation-induced scission of plasmid DNA [Yoshioka et al., 1996] and formation of 8-oxodeoxyguanosine in calf thymus DNA exposed to ONOO⁻ [Fiala et al., 1996]. EGCG inhibited stomach tumorigenesis in rodents exposed to N-methyl-N'-nitro-N-nitrosoguanidine [Yamane et al., 1995].

Most recently, we found that EGCG had the most antioxidant activity of several phenolic phytochemicals tested, as evaluated by the capacity of the test substances to scavenge the stable free radical, α , α -diphenyl- β -picrylhydrazyl [Johnson and Loo, 2000]. Moreover, EGCG inhibited oxidative damage to DNA that was induced by incubating cells briefly with either H₂O₂ or ONOO⁻. However, we did not study if EGCG inhibits the possible adverse effect of •NO on DNA and cell viability. Therefore, we determined if •NO damages DNA and induces cell cycle arrest and/or apoptosis. Most importantly, we evaluated the efficacy of EGCG to protect cells against •NO.

MATERIALS AND METHODS

Materials

U937 human pro-monocytic cells were purchased from ATCC (Rockville, MD). RPMI 1640 cell culture medium and additives, EGCG, camptothecin, propidium iodide (PI), low and normal melting point agarose were purchased from Sigma Chemical Co. (St. Louis, MO). S-

nitrosoglutathione (GSNO) was purchased from Calbiochem (La Jolla, CA). General reagents and frosted microscope slides were purchased from Fisher Scientific Co. (Pittsburgh, PA). The annexin V-FITC binding assay kit was purchased from PharMingen (San Diego, CA).

Cell Culture and Treatment

U937 cells were grown in RPMI 1640 medium that was supplemented with 10% fetal bovine serum, 1% glutamine, 1% penicillin-streptomycin (10,000 U/ml), and 0.2% amphotericin B (250 μ g/ml). The cells were maintained in a humidified incubator (95% air/5% CO₂) set at 37°C.

Cells (5×10^5 /ml) were co-treated with 0 or 500 μ M GSNO and 0, 20 μ M (9 μ g/ml), 30 μ M (14 μ g/ml) or 40 μ M (18 μ g/ml) EGCG, and then placed inside the humidified incubator (time point zero). Because GSNO has a half-life of several hours and thus generates a steady flux of •NO during the course of incubation [Kroncke and Kolb-Bachofen, 1999], the U937 cells receiving the EGCG treatment were dosed with an additional 0, 9, 14, or 18 μ g/ml of EGCG every 2 h throughout the experiment. After 4–6 h of total incubation time, the cells were harvested for analysis. In some experiments, camptothecin (1 μ g/ml), a known inducer of cell cycle arrest and apoptosis [Johnson et al., 1997], was used as a positive control.

Detection of Single-Strand Breaks in Cellular DNA

To detect DNA damage (in the form of single-strand breaks) in the U937 cells, alkaline microgel electrophoresis (i.e., comet assay) was performed under dim light [Singh et al., 1988]. Briefly, after exposure of cells (5×10^4) to GSNO for 4 h in the absence and presence of EGCG, the U937 cells were washed and then resuspended in 1% low melting point agarose dissolved in phosphate-buffered saline (PBS). Then, 75 μ l of this mixture was pipetted onto a frosted microscope slide that had been pre-coated with 75 μ l of 1% normal melting point agarose dissolved in PBS. After immediately placing a glass cover slip over the slide, the agarose/cell mixture was allowed to congeal on ice for 10 min. After removing the cover slip, the slide was immersed in ice-cold lysis solution (2.5 M NaCl, 100 mM disodium EDTA, 10 mM Tris, 10% DMSO, and 1% Triton X-100) for 1 h. Next, slides were placed in a submarine gel electrophoresis unit

containing 300 mM NaOH and 1 mM EDTA, pH 13, for 40 min, before being electrophoresed at 20 V (300 mA) for 20 min. Following electrophoresis, the slides were immersed in a neutralizing buffer (0.4 M Tris-HCl, pH 7.5), before applying 60 μ l of 2 μ g/ml ethidium bromide to stain the DNA.

Slides were viewed on an Olympus IX-70 inverted fluorescence microscope. Each of 50 randomly-selected nucleoids per slide was scored on an arbitrary scale of 0–4 (i.e., ranging from no damage to extensive damage of DNA), which is a reflection of the comet tail span. Hence, the data are reported as the comet score with a range of 0–200. Despite being subjective, this way of scoring comets compares acceptably with computer imaging analysis [McCarthy et al., 1997].

DNA Cell Cycle Analysis

To quantitatively determine the distribution of cells in the cell cycle, flow cytometric analysis of cellular DNA content was performed [Gong et al., 1994]. After exposure to GSNO for 6 h in the absence and presence of EGCG, U937 cells (2×10^6) were washed twice with ice-cold PBS ($\text{Ca}^{2+}/\text{Mg}^{2+}$ -free). The cells were then fixed with 2 ml of ice-cold 70% ethanol and incubated overnight at -20°C . Next, the cells were washed with the ice-cold PBS to remove the ethanol. The cell pellet was resuspended in 500 μ l of the PBS, mixed with 1 ml of DNA extraction buffer (0.2 M Na_2PO_4 and 0.1 M citric acid, pH 7.8), and incubated at room temperature for 5 min. After centrifugation at 300g for 8 min, the cell pellet was resuspended in 1 ml of a solution containing 0.5 μ g/ml RNase and 0.08 mg/ml PI for subsequent incubation in the dark at room temperature for 30 min. The PI fluorescence associated with DNA was measured on a Becton-Dickinson FACSCalibur flow cytometer with excitation at 488 nm and emission at 585 nm using the FL2 channel. The percentage of nuclei in each of the cell cycle phases (G_1 , S, G_2/M) was calculated from the standard DNA histograms. Additionally, a sub- G_1 peak on the DNA histograms was interpreted as an apoptotic cell population having a lowered DNA content [Darzynkiewicz et al., 1992].

Examination of the Cells for Morphological Signs of Apoptosis

To assess U937 cells for morphological signs of apoptosis, i.e., membrane blebbing and for-

mation of apoptotic bodies [Samali et al., 1996], the cells were examined on a phase-contrast light microscope. After exposure to GSNO for 6 h in the absence and presence of EGCG, 200 randomly selected cells per treatment were scored for presence of the morphological markers of apoptosis. The percentage of apoptotic cells was then calculated from the total number of cells scored.

Determination of Annexin V-FITC Binding as Another Marker of Apoptosis

In the beginning stages of apoptosis, there is translocation of phosphatidylserine (PS) from the inner to outer leaflet of the plasma membrane. This apoptotic event was determined with an annexin V-FITC binding assay kit following the manufacturer's instructions. The annexin V-FITC binds to the PS, thus giving a fluorescence signature to apoptotic cells [Zhang et al., 1997].

After 6 h of exposure to GSNO in the absence or presence of EGCG, U937 cells (1×10^6) were washed twice with ice-cold PBS. The cell pellet was resuspended in 1 ml of 1X binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl_2). To 100 μ l of this cell suspension, 5 μ l of Annexin V-FITC and 10 μ l of PI (0.5 μ g) were added and mixed. After 15 min of incubation in the dark, 400 μ l of 1X binding buffer were added and mixed. The annexin V-FITC/PI fluorescence was measured on the FACSCalibur flow cytometer, using the FL1 and FL2 channels, respectively.

Statistical Analysis of Data

Where applicable, data were analyzed by analysis of variance (ANOVA) and Fisher's Protected Least Significant Differences Test using a commercial computer program (SYS STAT, Chicago, IL).

RESULTS

As shown in Figure 1, exposing U937 cells to GSNO (or more precisely, the $\bullet\text{NO}$ generated from GSNO) for 4 h did not induce single-strand breaks in DNA, as assessed by the comet assay. The comet scores for GSNO-treated and untreated cells were 20 ± 7 and 15 ± 3 , respectively. Moreover, no increase in DNA damage was detected in cells that were exposed to GSNO for shorter time periods (data not shown). In contrast, EGCG, which was anticipated only to

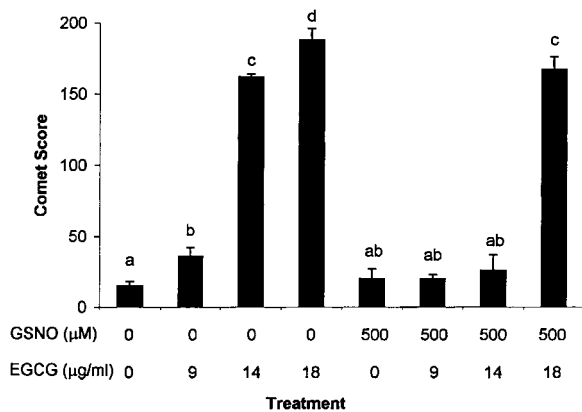


Fig. 1. Assessment of single strand breaks in U937 monocytes after incubation with either GSNO, EGCG, or GSNO/EGCG. Cells were treated with 500 μM GSNO alone and also with 9, 14, or 18 μg/ml of EGCG alone. Other cells were co-treated with GSNO and either 9, 14, or 18 μg/ml of EGCG. However, after the first 2 h of incubation, these cells were dosed with an additional 9, 14, or 18 μg/ml of EGCG. After 4 h of total incubation for all the cell samples, the extent of DNA single-strand breaks was assessed by the comet assay, as described in materials and methods. Data are the mean ± SEM of three different experiments. Values not sharing the same letter are significantly different from one another ($P < 0.05$).

inhibit any GSNO-induced DNA damage, actually promoted DNA damage in a concentration-dependent manner. Dosing U937 cells with 9, 14, and 18 μg/ml of EGCG resulted in comet scores of 36 ± 6 , 162 ± 2 , and 188 ± 8 , respectively. By interpreting other data in Figure 1 from a totally different perspective, it seems that 500 μM GSNO inhibited the DNA damage that was induced by dosing the cells with 9, 14, or 18 μg/ml of EGCG.

Although EGCG by itself induced DNA damage in U937 cells, cell cycle arrest was not detected. The percentages of cells from EGCG-treated and untreated control samples in the G_1 -phase (Fig. 2) and G_2/M -phase (Fig. 3) of the cell cycle were not different. Moreover, EGCG treatment did not affect the percentage of cells in the S-phase (Fig. 4).

In contrast, despite not increasing DNA single-strand breaks in U937 cells, GSNO caused cell cycle arrest. There was $82 \pm 4\%$ of GSNO-treated cells, compared to only $39 \pm 1\%$ of untreated control cells, present in the G_1 -phase (Fig. 2). Accordingly, only $11 \pm 4\%$ of GSNO-treated cells, compared to $44 \pm 1\%$ of untreated control cells, were found in the S-phase (Fig. 4).

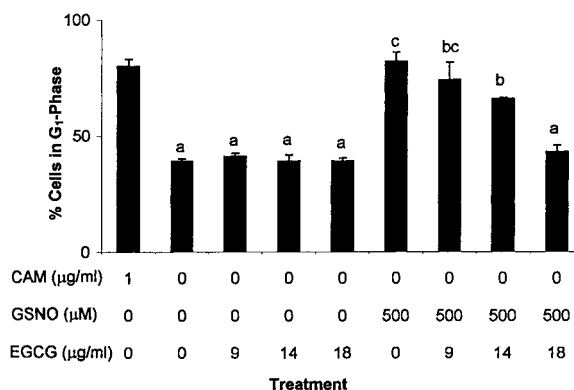


Fig. 2. Percentage of U937 monocytes in the G_1 -phase of the cell cycle after incubation with either GSNO, EGCG, or GSNO/EGCG. Cells were treated with 500 μM GSNO alone and also with 9, 14, or 18 μg/ml of EGCG alone. Other cells were co-treated with GSNO and either 9, 14, or 18 μg/ml of EGCG. However, at the 2 and 4-h incubation time points, these cells were dosed with an additional 9, 14, or 18 μg/ml of EGCG. Cells treated with camptothecin (1 μg/ml), a known inducer of apoptosis, were used as a positive control. After 6 h of total incubation for all the cell samples, DNA cell cycle analysis was performed by flow cytometry, as described in materials and methods. Data are the mean ± SEM of four different experiments. Values not sharing the same letter are significantly different from one another ($P < 0.05$).

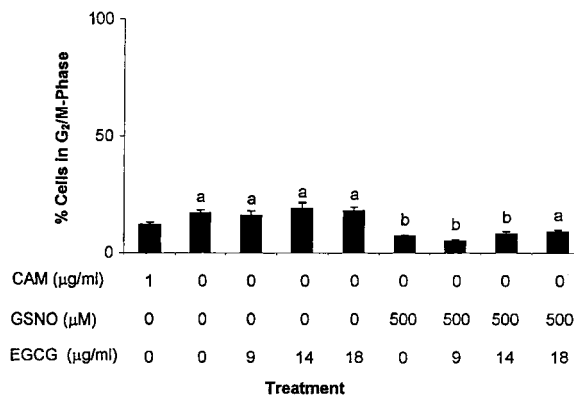


Fig. 3. Percentage of U937 monocytes in the G_2/M -phase of the cell cycle after incubation with either GSNO, EGCG, or GSNO/EGCG. Cells were treated with 500 μM GSNO alone and also with 9, 14, or 18 μg/ml of EGCG alone. Other cells were co-treated with GSNO and either 9, 14, or 18 μg/ml of EGCG. However, at the 2 and 4-h incubation time points, these cells were dosed with an additional 9, 14, or 18 μg/ml of EGCG. Cells treated with camptothecin (1 μg/ml), a known inducer of apoptosis, were used as a positive control. After 6 h of total incubation for all the cell samples, DNA cell cycle analysis was performed by flow cytometry, as described in materials and methods. Data are the mean ± SEM of four different experiments. Values not sharing the same letter are significantly different from one another ($P < 0.05$).

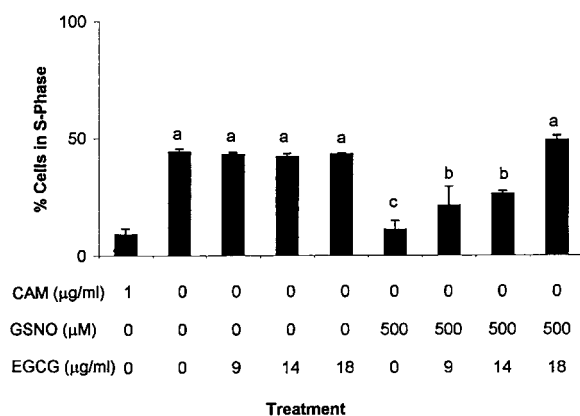


Fig. 4. Percentage of U937 monocytes in the S-phase of the cell cycle after incubation with either GSNO, EGCG, or GSNO/EGCG. Cells were treated with 500 µM GSNO alone and also with 9, 14, or 18 µg/ml of EGCG alone. Other cells were co-treated with GSNO and either 9, 14, or 18 µg/ml of EGCG. However, at the 2 and 4-h incubation time points, these cells were dosed with an additional 9, 14, or 18 µg/ml of EGCG. Cells treated with camptothecin (1 µg/ml), a known inducer of apoptosis, were used as a positive control. After 6 h of total incubation for all the cell samples, DNA cell cycle analysis was performed by flow cytometry, as described in materials and methods. Data are the mean \pm SEM of four different experiments. Values not sharing the same letter are significantly different from one another ($P < 0.05$).

As further revealed by the data, co-treating or dosing the GSNO-treated cells with EGCG resulted in a lower percentage of cells in the G₁-phase (Fig. 2) and a higher percentage of cells in the S-phase (Fig. 4). Doses of 9 and 14 µg/ml of EGCG had only slight effects, but 18 µg/ml of EGCG had a very notable effect because the percentage of cells in the G₁-phase ($43 \pm 3\%$) and S-phase ($49 \pm 2\%$) were similar to their respective control values. Additionally, a distinct sub-G₁ peak, indicative of an apoptotic cell population with hypodiploid DNA content, was observed on the flow cytometric DNA histograms for U937 cells that were exposed to GSNO. When translated into numerical values, GSNO-treated cells had a sub-G₁ cell population of $14 \pm 3\%$ compared to only $1 \pm 0\%$ in control cells (Fig. 5). Treatment of cells with EGCG alone did not increase the sub-G₁ cell population over the control. However, dosing U937 cells with 9, 14, or 18 µg/ml of EGCG after GSNO treatment resulted in sub-G₁ populations of $5 \pm 2\%$, $5 \pm 1\%$, and $2 \pm 0\%$, respectively.

To confirm the ability of EGCG to protect U937 cells against the cytotoxic effects of GSNO, other markers of apoptosis such as cell

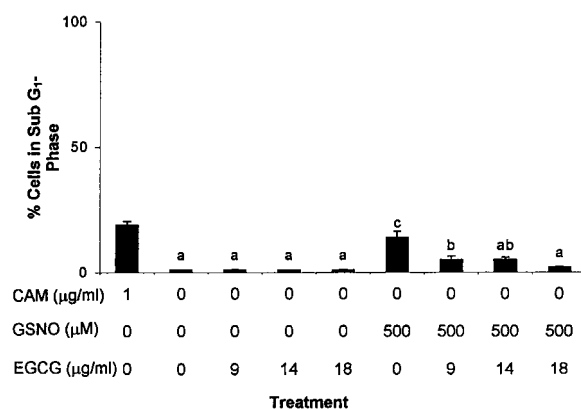


Fig. 5. Percentage of U937 monocytes in the sub-G₁-phase of the cell cycle after incubation with either GSNO, EGCG, or GSNO/EGCG. Cells were treated with 500 µM GSNO alone and also with 9, 14, or 18 µg/ml of EGCG alone. Other cells were co-treated with GSNO and either 9, 14, or 18 µg/ml of EGCG. However, at the 2 and 4-h incubation time points, these cells were dosed with an additional 9, 14, or 18 µg/ml of EGCG. Cells treated with camptothecin (1 µg/ml), a known inducer of apoptosis, were used as a positive control. After 6 h of total incubation for all the cell samples, DNA cell cycle analysis was performed by flow cytometry, as described in materials and methods. Data are the mean \pm SEM of four different experiments. Values not sharing the same letter are significantly different from one another ($P < 0.05$).

morphological changes and annexin V-FITC binding were considered as well. In examining cells for morphological signs of apoptosis (Fig. 6), membrane blebbing and presence of apoptotic bodies (Fig. 7) were observed in $30 \pm 4\%$ of the U937 cells exposed to GSNO, compared to only $4 \pm 1\%$ in control cells. EGCG alone did not produce such morphological changes, and again protected the cells against the cytotoxic effect of GSNO. Only $3 \pm 1\%$, $6 \pm 2\%$, and $6 \pm 1\%$ of the GSNO-treated cells that were dosed with 9, 14, and 18 µg/ml of EGCG, respectively, showed the morphological signs of apoptosis.

Annexin V-FITC binding data generated by flow cytometry (Fig. 8) show that only $3 \pm 0\%$ of control cells bound annexin V-FITC, whereas $48 \pm 3\%$ of GSNO-treated cells bound annexin V-FITC, as exemplified in Figure 9. Treating cells with EGCG alone did not increase the percentage of cells binding annexin V-FITC. However, dosing U937 cells with 9 or 14 µg/ml of EGCG resulted in a lower percentage of cells binding annexin V-FITC ($28 \pm 6\%$ and $23 \pm 4\%$, respectively). Dosing GSNO-treated cells with 18 µg/ml of EGCG resulted in only $5 \pm 1\%$ of the cells binding annexin V-FITC.

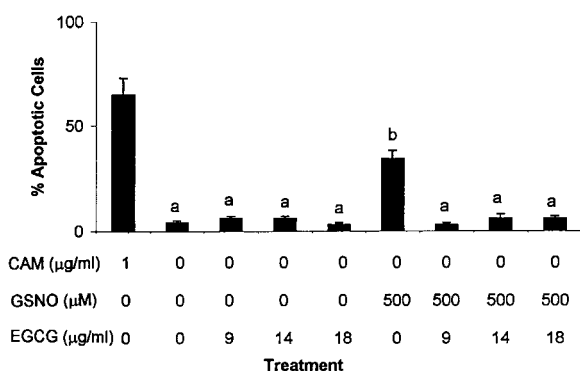


Fig. 6. Lowering by EGCG of the number of GSNO-treated U937 monocytes showing morphological signs of apoptosis. Cells were treated with 500 μM GSNO alone and also with 9, 14, or 18 μg/ml of EGCG alone. Other cells were co-treated with GSNO and either 9, 14, or 18 μg/ml of EGCG. However, at the 2 and 4-h incubation time points, these cells were dosed with an additional 9, 14, or 18 μg/ml of EGCG. Cells treated with camptothecin (1 μg/ml), a known inducer of apoptosis, were used as a positive control. After 6 h of total incubation for all the cell samples, cells were scored for morphological signs of apoptosis, as described in materials and methods. Data are the mean ± SEM of three different experiments. Values not sharing the same letter are significantly different from one another ($P < 0.05$).

DISCUSSION

The basis for the current study emanated from our most recent but limited study [Johnson and Loo, 2000], where it is reported that EGCG inhibited both H_2O_2 -induced and $ONOO^-$ -induced oxidative damage to cellular DNA. To expand this previous work, we focused on $\bullet NO$ in studying whether this nitrogen free radical could not only induce DNA damage but also cell cycle arrest and apoptosis. We simultaneously evaluated the ability of EGCG to inhibit the adverse effects of $\bullet NO$. We originally hypothesized that $\bullet NO$ would cause single-strand breaks in DNA. Moreover, we hypothesized that if EGCG scavenged $\bullet NO$ and prevented the structural damage to DNA, cell cycle arrest and/or apoptosis would not occur. In other words, if EGCG decreased the number of cells in a state of cell cycle arrest or apoptosis when they are exposed to $\bullet NO$, this would more likely mean that EGCG inhibited structural damage to DNA rather than EGCG interfering with steps or components of the cell cycle and apoptosis.

To expose them to $\bullet NO$, U937 cells were incubated at 37°C with GSNO. During incubation, GSNO spontaneously decomposes to produce $\bullet NO$ for several hours [Kroncke and Kolb-

Bachofen, 1999]. After readily diffusing across lipid bilayers of cell membranes [Subczynski et al., 1996], $\bullet NO$ travels freely within the cell and reacts with other molecules to generate additional types of RNS. For example, an important reaction of $\bullet NO$ at physiological pH is its oxidation by molecular oxygen to yield nitrous anhydride (N_2O_3) [Marletta, 1988; Lewis et al., 1995]. Another major fate of $\bullet NO$ is its reaction with O_2^- to yield $ONOO^-$ [Huie and Padmaja, 1993]. Thus, it is possible that any adverse effects of GSNO-derived $\bullet NO$ may be attributed partly to N_2O_3 , $ONOO^-$, or perhaps other downstream active products. Both N_2O_3 and $ONOO^-$ are potent nitrosation agents, capable of chemically modifying the structure of DNA and causing temporary single-strand breaks in DNA [Burney et al., 1999]. That is, nitrosylation of primary amines on the purine and pyrimidine bases of DNA leads to their deamination via diazonium ion formation. Hydrolysis of the diazonium ion completes the reaction sequence, resulting in the replacement of an amino group by a hydroxyl group. Such chemically modified sites on DNA are cleaved by DNA glycosylases, giving rise to temporary single-strand breaks as nucleotide excision repair occurs [Yu et al., 1999]. Furthermore, $ONOO^-$ can cause oxidative damage to DNA. Upon protonation, $ONOO^-$ is transformed into peroxyntrous acid that can exist as an excited intermediate having hydroxyl radical-like oxidative potential [Aust and Eveleigh, 1999]. Indeed, $ONOO^-$ may actually generate $\bullet OH$ [Beckman et al., 1990] that attacks the deoxyribose moiety of DNA in causing strand breaks [Hiramoto et al., 1996].

It has been reported [Green et al., 1996] that GSNO can cause single-strand breaks in cellular DNA, as assessed by the comet assay. As a discrepancy, we were unable to detect an increase in DNA single-strand breaks in U937 cells exposed to GSNO for 4 h or less. While $\bullet NO$ nitrosylatively modifies the structure of DNA [Burney et al., 1999] and suppresses DNA synthesis by inhibiting ribonucleotide reductase [Lepoivre et al., 1991], it is inconclusive if $\bullet NO$ directly causes strand breaks in DNA [Nguyen et al., 1992; Tamir et al., 1996]. In contrast, exposing Jurkat T-lymphocytes for 30 min to 3-morpholininosydnonimine (SIN-1), which thermally decomposes to generate $ONOO^-$, resulted in a marked increase in DNA single-strand breaks [Johnson and Loo,

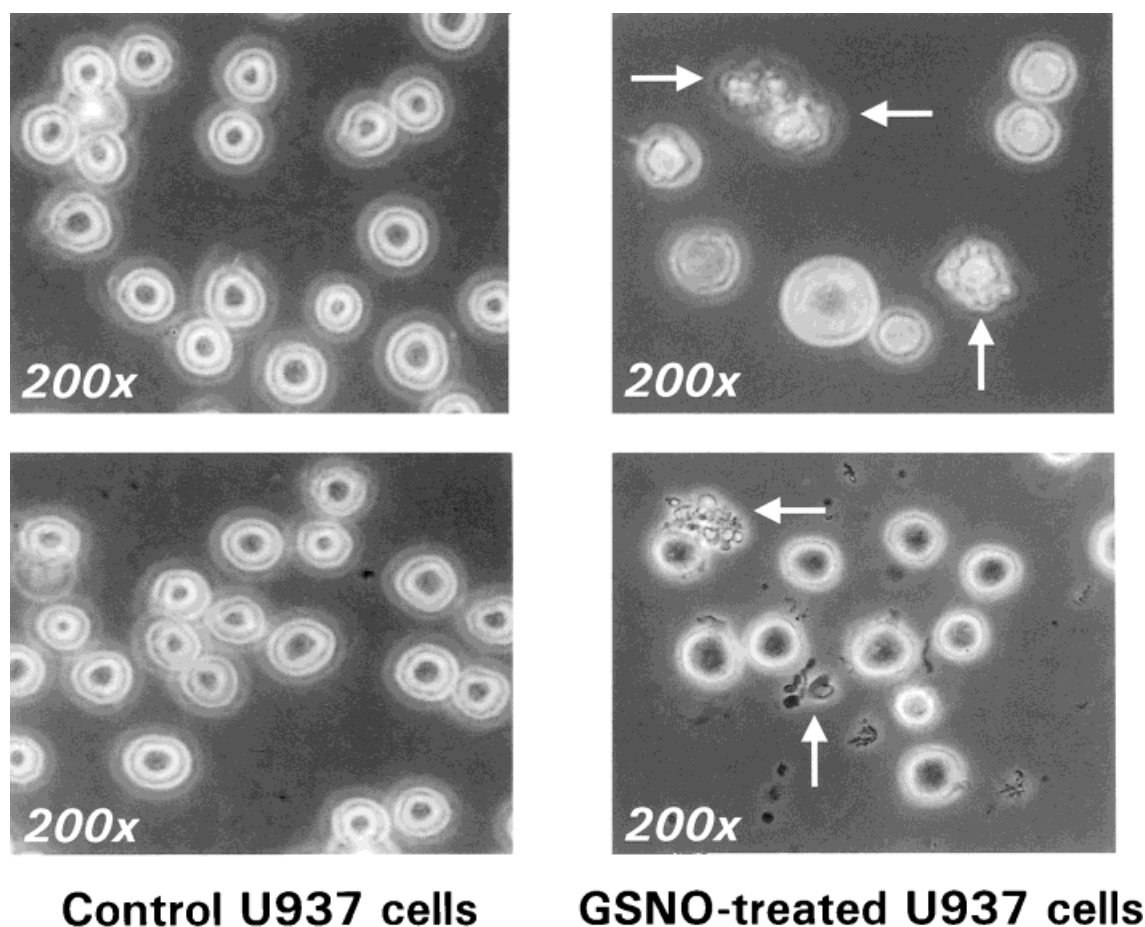


Fig. 7. Representative photos of apoptotic morphology induced in U937 cells after exposure to 500 μM GSNO for 6 h. Arrows in the upper-right photo point to membrane blebbing. Arrows in the lower-right photo point to apoptotic bodies.

2000]. Therefore, it seems that ONOO^- was not generated in sufficient amounts, from the reaction between GSNO-derived $\bullet\text{NO}$ and existing O_2^- , to significantly increase DNA single-strand breaks when U937 cells were exposed to GSNO. Instead of GSNO-derived $\bullet\text{NO}$ causing direct DNA damage, it seems more probable that the $\bullet\text{NO}$ generated from GSNO immediately reacted to form N_2O_3 or perhaps other derivatives, which then nitrosylatively modify the structure of DNA. Hence, it is suggested that $\bullet\text{NO}$ mediated its cellular effects by nitrosylatively modifying the chemical structure of DNA rather than by inducing direct single-strand breaks in DNA as $\bullet\text{OH}$ is capable of doing [Hiramoto et al., 1996].

Cells that sustain extensive covalent modification or damage to DNA typically arrest in the G_1 -phase of the cell cycle, thus stopping DNA synthesis [Canman et al., 1994]. During this

time, the cells attempt to repair the DNA before progressing into the S-phase. Apparently, the probable nitrosylative modification of DNA caused by GSNO, or its degradative products, in U937 cells was extensive enough to induce cell cycle arrest. An increased number of cells were found arrested in the G_1 -phase. Dosing of GSNO-treated U937 cells with EGCG reduced the number of cells in G_1 -phase arrest. EGCG likely scavenged the $\bullet\text{NO}$ or other GSNO-derived active products, thereby negating structural modification of DNA that may have initiated the cell cycle arrest. In support of this view, it is well established that phenolic phytochemicals like EGCG scavenge $\bullet\text{NO}$ [van Acker et al., 1995] and ONOO^- [Haenen et al., 1997]. Pre-incubating DNA bases with either EGCG or closely related substances (catechin and epicatechin) inhibited the formation of hypoxanthine and xanthine that was caused

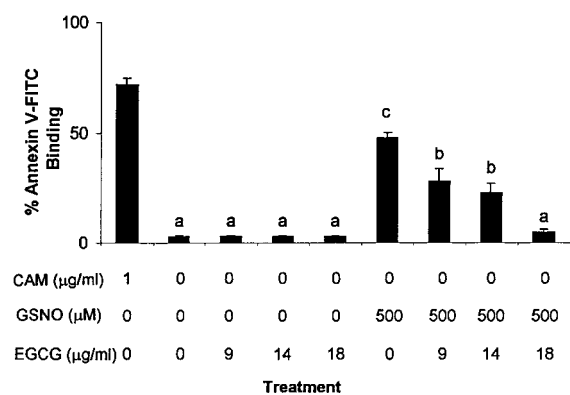


Fig. 8. Lowering by EGCG of the number of GSNO-treated U937 monocytes binding annexin V-FITC. Cells were treated with 500 μM GSNO alone and also with 9, 14, or 18 μg/ml of EGCG alone. Other cells were co-treated with GSNO and either 9, 14, or 18 μg/ml of EGCG. However, at the 2 and 4-h incubation time points, these cells were dosed with an additional 9, 14, or 18 μg/ml of EGCG. Cells treated with camptothecin (1 μg/ml), a known inducer of cell cycle arrest and apoptosis, were used as a positive control. After 6 h of total incubation for all the cell samples, the degree of cell surface binding of annexin V-FITC was determined by flow cytometry as described in materials and methods. Data are the mean ± SEM of four different experiments. Values not sharing the same letter are significantly different from one another ($P < 0.05$).

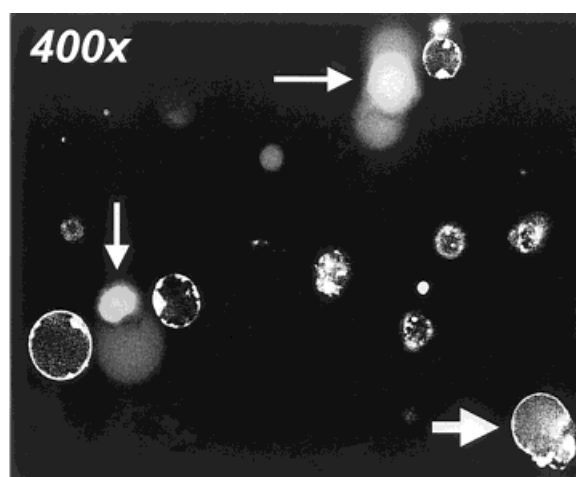
by the acidic nitrite-dependent deamination of adenine and guanine, respectively [Oldreive et al., 1998]. In another relevant study, EGCG inhibited ONOO⁻-induced formation of 8-oxo-deoxyguanosine in calf thymus DNA [Fiala et al., 1996].

Besides affecting DNA directly, it is possible that the •NO derived from GSNO also had other effects that promoted cell cycle arrest in the U937 cells. •NO inhibits iron-sulfur proteins such as ribonucleotide reductase [Lepoivre et al., 1991], an enzyme that catalyzes biosynthesis of the deoxynucleotide building blocks of DNA. Hence, a lowered DNA content in cells might also contribute to the initiation of cell cycle arrest. Moreover, •NO inhibits numerous sulfhydryl enzymes, such as glyceraldehyde-3-phosphate dehydrogenase [Padgett and Whorton, 1997]. Inhibition of this specific enzyme in glycolysis could limit the amount of energy needed by cells to synthesize DNA.

In any event, exposing U937 cells to GSNO led eventually to apoptosis, which was evaluated by examining several standard markers. Flow cytometric analysis of GSNO-treated cells revealed a substantial sub-G₁ peak on the DNA cell cycle histograms, indicating a population of apoptotic cells with less DNA content due to the action of endonucleases that fragment the DNA [Enari et al., 1998]. Upon microscopic examination, membrane blebbing and small apoptotic bodies were seen in GSNO-treated cell samples. Furthermore, exposing U937 cells to GSNO resulted in a higher number of the cells binding annexin V-FITC. These findings are consistent with other research [Brockhaus and Brune, 1998] that additionally reported the activation of caspases, which are cysteine proteases that



Control U937 cells



GSNO-treated U937 cells

Fig. 9. Representative photos of increased annexin V-FITC binding by U937 cells after exposure to 500 μM GSNO for 6 h. Arrows in the right photo point to cells that have also taken up propidium iodide, indicating that they are in the late stage of apoptosis or already dead.

initiate the execution phase of apoptosis [Wilson, 1998]. In agreement with its ability to reduce the number of GSNO-treated cells in G₁-phase arrest, EGCG also reduced the number of GSNO-treated cells undergoing apoptosis. In other words, dosing GSNO-treated cells with EGCG eliminated the sub-G₁ peak appearing on the DNA cell cycle histograms, reduced the number of cells binding annexin V-FITC, and also prevented membrane blebbing and formation of apoptotic bodies.

The mechanism by which EGCG lowered the numbers of GSNO-treated U937 cells in a state of either cell cycle arrest or apoptosis can be deduced. In the presence of certain substances (e.g., GSNO) that can generate RNS, EGCG has antioxidant or radical scavenging effects. That is, phenolic phytochemicals such as EGCG scavenge both •NO [van Acker et al., 1995] and ONOO⁻ [Haenen et al., 1997]. Therefore, in addition to scavenging any ROS present, EGCG may have worked largely by scavenging the •NO produced from GSNO and any ONOO⁻ formed from the reaction of •NO and existing O₂⁻. As such, the phenolic structure of EGCG may be nitrosated by the GSNO-derived RNS. There is evidence for the nitrosation of EGCG by ONOO⁻ [Fiala et al., 1996]. With the RNS scavenged by the EGCG, DNA would not be attacked, and thus, both cell cycle arrest and apoptosis would not occur.

Although EGCG was predicted and shown to counteract the cytotoxic effects of GSNO, EGCG had some seemingly contradictory effects. Incubating U937 cells with EGCG alone for 4 h resulted in significant DNA damage. While this may seem unusual, it is consistent with data in our most recent study [Johnson and Loo, 2000], where it is reported that Jurkat T-lymphocytes have increased DNA single-strand breaks after being incubated with either EGCG or quercetin. Furthermore, other studies have established that phenolic phytochemicals can actually induce DNA single-strand breaks as determined by the comet assay. For instance, increased DNA single-strand breaks were found in Caco-2 colonic adenocarcinoma, HepG2 hepatoma, and HeLa cervical carcinoma epithelial cells when these cell lines were incubated with either quercetin, myricetin, or silymarin [Duthie et al., 1997].

The pro-oxidant effects of phenolic phytochemicals appear to be due to the presence of metal ions, which can facilitate the formation of

ROS [Halliwell and Gutteridge, 1990]. For instance, quercetin induced DNA damage in isolated rat liver nuclei in the presence of iron or copper [Sahu and Washington, 1991]. Both EGCG and quercetin induced strand breaks in plasmid DNA, but this damage was prevented when the metal chelator, diethylenetriamine pentaacetic acid, was present during incubation [Ohshima et al., 1998]. Considering that cell culture media contain metal ions and the recent report [Long et al., 2000] that adding EGCG to cell culture media results in the generation of H₂O₂, the DNA damage induced by EGCG could be caused by hydroxyl radicals, that form when H₂O₂ reacts with metal ions [Halliwell and Gutteridge, 1990].

In light of the above discussion, it seems that EGCG can have either antioxidant or pro-oxidant effects on DNA in U937 cells depending on the experimental conditions. The capacity of phenolic phytochemicals like EGCG to exert such opposite effects has been demonstrated by others as well [Duthie et al., 1997; Aherne and O'Brien, 1999]. This would explain why EGCG protected the cells against GSNO-mediated apoptosis on the one hand, but induced DNA strand breaks on the other hand. More specifically, in the presence of pro-oxidants or substances such as GSNO that generate free radicals, EGCG is probably a free radical scavenger. As such, EGCG was able to scavenge GSNO-derived •NO or other active products that could have caused structural modification of DNA (not to be confused with single-strand breaks) or other adverse effects leading to the induction of apoptosis. As a result of reducing the number of GSNO-treated U937 cells undergoing apoptosis, EGCG protected the cells so that they remained in cell cycle progression as indicated by the higher number of cells in the S-phase compared to U937 cells not fortified with EGCG prior to GSNO exposure.

In striking contrast, in the absence of any added pro-oxidants but the presence of trace amounts of metal ions in cultures of U937 cells, EGCG is probably a pro-oxidant or can promote the formation of free radicals. As such, EGCG was able to cause single-strand breaks in DNA although apparently not to an extent sufficient to initiate apoptosis. Taken together, these findings suggest that the relatively low concentrations of EGCG (20–30 μM) did not induce DNA single-strand breaks in U937 cells when GSNO (500 μM) was also present, as supported

by the data in Figure 1. In interpreting these data from a completely different perspective, an unexpected effect of GSNO in EGCG-treated U937 cells can be addressed. Apparently, by donating •NO to react with EGCG, GSNO inhibited induction of DNA single-strand breaks caused by EGCG. It is known that under certain conditions •NO has antioxidant effects. For example, •NO can protect cells against the cytotoxic effects of H₂O₂ [Wink et al., 1999] and can inhibit the oxidation of lipoproteins [Goss et al., 1999]. Lastly, our findings suggest that DNA damage (single-strand breaks) was not the cellular event that triggered apoptosis in GSNO-treated U937 cells. Some other triggering event (e.g., perhaps structural or covalent modification of DNA) was responsible, and EGCG was able to negate it.

As already discussed, the DNA damage caused by EGCG in U937 cells was apparently not sufficient to trigger either cell cycle arrest or apoptosis under our experimental conditions. In contrast, using some other cell lines and different experimental conditions, EGCG induced G₁-phase arrest [Liang et al., 1999], inhibited DNA synthesis or the number of cells in the S-phase [Otsuka et al., 1998], and induced G₂/M arrest [Okabe et al., 1999]. Furthermore, EGCG induced apoptosis in fibroblasts [Chen et al., 1998] and three different prostate cancer cell lines [Paschka et al., 1998]. Other studies have reported cell cycle arrest or apoptosis in other types of cells incubated either with higher doses of other phenolic phytochemicals or for longer periods of time. Along this line, genistein [Spinozzi et al., 1994] and apigenin [Lepley and Pelling, 1997] induced cell cycle arrest in Jurkat T-lymphocytes and human fibroblast cells, respectively. Additionally, phenolic phytochemicals such as genistein [Spinozzi et al., 1994], apigenin and quercetin [Plaumann et al., 1996; Richter et al., 1999], fisetin and kaempferol [Richter et al., 1999], and luteolin [Plaumann et al., 1996] induced apoptosis. Hence, differences in concentrations of the phenolic phytochemicals, cell lines used, and incubation times may explain the discrepancy between our results and that of others.

In conclusion, by likely scavenging GSNO-derived •NO or its downstream products and thus preventing structural modification of DNA or possibly other adverse cellular effects, EGCG protected cells against the cytotoxic effects of GSNO as indicated by the lower number of

GSNO-treated cells in a state of cell cycle arrest and apoptosis.

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