Involvement of Calcium-Depended Protein Kinase C in Low Dose γ-Rays-induced Increase of Glutathione in RAW 264.7 Cells

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SUMMARY

We examined the mechanisms of the elevation of glutathione level induced in macrophage-like RAW 264.7 cells by low doses of γ-rays. The level increased soon after exposure of the cells to 50 cGy of γ-rays, peaked between 3 hr and 6 hr, and returned almost to the time 0 value by 24 hr post-irradiation. Doses between 25 and 100 cGy significantly increased the glutathione level at 4 hr post-irradiation. However, there was no significant elevation at doses of more than 100 cGy or less than 25 cGy. When the effect of dose rate was examined at a constant absorbed dose of 50 cGy, dose rates of more than 50 cGy/min significantly increased the GSH level at 4 hr post-irradiation. It was also shown that the elevation of glutathione level in cells irradiated with low doses of γ-rays followed the induction of mRNA coding for γ-glutamylcysteine synthetase (γ-GCS), a rate-limiting enzyme of the de novo glutathione synthesis pathway. When the cells were exposed to the radiation in the presence of genistein, calphostin C or nifedipine, the elevations of glutathione and γ-GCS mRNA expression were both mostly blocked. EGTA also strongly inhibited these elevations. These results suggest that the tyrosine kinase, calcium channel, and protein kinase C activities play an essential role in the low-dose-radiation-induced elevation of cellular glutathione.

Keywords: Low-dose γ-rays, Glutathione, Protein kinase C, Calcium-channel

INTRODUCTION

Low doses of ionizing radiation induce various effects, including radioadaptive response (1-3), activation of immune function (4-6), an increase in life-span (7), and enhancement of resistance to high-dose radiation (8, 9). The mechanisms of these effects have still not been established.

In our previous studies, it was found that low doses (25-50 cGy) of radiation elevated the glutathione level in mouse organs, such as liver, pancreas and brain (10), and these elevations were accompanied by increased activities of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme for de novo glutathione synthesis (GSH), and glutathione reductase (GR) (EC 1.6.4.2), an enzyme involved in the regeneration of GSH from oxidized glutathione (GSSG). It was subsequently confirmed that this phenomenon is a post-transcriptional event; the elevation of glutathione levels in these organs follows the induction of mRNAs coding for γ-GCS and GR (11, 12).

In this study, we investigated in detail the elevation of cellular glutathione level induced by low doses of γ-rays in cultured cells, in order to elucidate further the mechanisms involved in this phenomenon.

MATERIALS AND METHODS

Materials

GSH, GSSG, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and ethylene glyco-bis [β-aminoethyl ether]-N,N,N',N'-tetraacetic acid were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). β-NADPH and GR (120 U/ml) were obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan). L-Buthionine-SR-sulfoximine, nifedipine, genistein and calphostin C were from Sigma (St. Louis, MO, U.S.A). All other reagents were of analytical grade.

Cell Cultures

Mouse macrophage-like RAW 264.7 cell was purchased from Riken Cell Bank (Tsukuba, Japan). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in a 5% CO₂/95% air atmosphere. Cells in the logarithmic phase were used throughout the experiment.

γ-Ray Irradiation

Cells were seeded in 6-well plates at a density of 5 x 10⁵ cells/well, incubated for 12 hr and then
irradiated with $\gamma$-rays from a $^{137}$Cs source (GAMMACELL 40, Nordin International, Inc., Canada). Thereafter, the cells were incubated for the specified time intervals.

**Inhibitors**

For the study on the mechanisms, various inhibitors were added to the culture medium 30 min prior to irradiation with $\gamma$-rays. The cells were then incubated for 4 hr after the radiation exposure. The inhibitors used in this experiment were a specific inhibitor of protein kinase C (PKC), calphostin C (CALP, 50 nM); an inhibitor of tyrosine-specific kinase, genistein (GEN, 25 µM); a calcium channel blocker, nifedipine (NIF, 50 µM); a chelator of calcium, glyco-bis [β-aminoethylether]-N,N,N',N'-tetracetic acid (EGTA, 1 mM); and an inhibitor of γ-GCS, L-buthionine-SR-sulfoximine (BSO, 0.5 mM). The concentrations used for the second messenger inhibitors including CALP, GEN and NIF were equal to the reported IC50 concentrations (13).

**Assay of Total Glutathione**

For the determination of intracellular glutathione levels, the cultured cells were washed 3 times with ice-cold phosphate-buffered saline (PBS) and then scraped from the dishes with a silicon rubber policeman and harvested in Eppendorf tubes. The cell pellet was suspended in PBS containing 5 mM EDTA, 0.01% digitonin and 0.25% sodium cholate. The cells were disrupted by sonication and the sonicate was centrifuged at 10,000 x g for 20 min. An aliquot of the supernatant was removed for protein assay. An equal volume of 10% TCA was added to another aliquot, and the solutions were kept for 30 min on ice. The acid-soluble fraction was obtained by centrifugation at 10,000 x g for 20 min. The fraction was subjected to total glutathione assay after repeated removals (5 times) of TCA with ether. Total glutathione (GSH + GSSG) content was measured by using a modified spectrophotometric technique (14). Briefly, each sample fraction was diluted 1:5, and a 25 µl aliquot was mixed with 250 µl of 1 mM DTNB, 733 µl of 0.3 mM NADPH, and 10 µl of GR (2 U/ml). The rate of change in absorbance was measured at 412 nm. Authentic GSH (0-25 µM) was analyzed in the same manner. The GSH concentration of each sample was calculated as nmol/mg protein. Protein was determined by the method of Lowry et al. (15) using bovine serum albumin as a standard.

**RNA Isolation and Northern Blot Analysis**

Expression of γ-GCS and GR mRNAs was analyzed by Northern blotting. Total RNA was isolated from the cells by means of the acid guanidium isothiocyanate-pheno-chloroform extraction method. The RNA was quantified spectrophotometrically at 260 nm (the ratio of A$_{260}$ nm to A$_{280}$ nm always exceeded 1.8), and 15 µg aliquots of total RNA were sized-fractionated by electrophoresis on a 1.0 % agarose gel (Nippon Gene, Toyama, Japan). RNA was then blotted onto nylon membrane using 0.02 M MOPS buffer (pH 7.0), and immobilized by UV cross-linking. The relative amounts of RNA were judged by hybridization with a mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Specific cDNA probes were obtained as follows. A mouse GR cDNA was a kind gift from Prof. Dieter Werner, German Cancer Research Center (16). Mouse γ-GCS and GAPDH cDNAs were synthesized by RT-PCR (Titan, Boehringer Mannheim, Mannheim) from mouse liver total RNA using oligo DNA primers for γ-GCS (5'-CACATCTACACCGAGTCA-3' and 5'-TTCGCTTTTTCTAAATCTGTA-3') and GAPDH (5'-TGAAGGTGTGTAACGATTGTCG-3' and 5'-CATGTAGGCTGAGGCCCACCAC-3'). cDNA was amplified (35 cycles, 94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min) and PCR products were sub-cloned into the pGEM-T vector (Promega, Madison, WI) for amplification. Hybridization was carried out in a solution consisting of 5 x SSPE (20 x SSPE = 3.6 M NaCl, 200 mM NaH$_2$PO$_4$, 20 mM EDTA, pH 7.4), 10 x Denhardt’s reagent (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA), 50% formamide, 1.4% sodium dodecyl sulfate (SDS), and 0.1 mg/ml herring sperm DNA with $^{32}$P-labeled probes at 42 °C. After hybridization, the membrane was washed with 6 x SSC (20 x SSC = 3 M NaCl, 0.3 M trisodium citrate) and 0.1% SDS at 42 °C for 30 min, 1 x SSC and 0.1% SDS at 55 °C for 30 min, and 0.1 x SSC and 0.1% SDS at 60 °C for 30 min. Quantitation was done with a laser image analyzer (Fujix BAS 2500, Fuji Film, Kanagawa). The membrane was also exposed to an X-ray film (Fuji HR-HA30, Fuji Film) with an intensifying screen at -80 °C.

**Statistical Analysis**

The statistical significance of differences was determined by using Student's $t$ test for comparison between two groups or two-way repeated measures analysis of variance (ANOVA) and Dunnett’s tests for multiple comparison, where appropriate. $P$ values of less than 0.05 were considered significant.
RESULTS

Changes in the intracellular glutathione level induced by low doses of \( \gamma \)-ray irradiation were examined in detail in RAW 264.6 cells. First, time-dependent change of the cellular glutathione level after irradiation with 50 cGy of \( \gamma \)-rays was examined. As shown in Figure 1, the total glutathione level (GSH + GSSG) in the cells started to increase soon after the irradiation, peaked between 3 hr and 6 hr, and returned almost to the time 0 value by 24 hr post-irradiation. Second, the dose dependence of the effect of \( \gamma \)-rays on the total glutathione level at 4 hr post-irradiation was investigated. Doses between 25 and 100 cGy significantly increased the glutathione level at 4 hr post-irradiation (Figure 2). However, there was no significant elevation at doses of more than 100 cGy or less than 25 cGy. Third, dose-rate effects of \( \gamma \)-rays on the glutathione level were examined at the fixed absorbed dose of 50 cGy. Dose rates of more than 50 cGy/min significantly increased the glutathione level at 4 hr post-irradiation (Figure 3).

![Figure 1. Changes in the total glutathione (GSH + GSSG) level in RAW 264.7 cells after \( \gamma \)-ray irradiation at a dose of 50 cGy](image)

Each point indicates the mean ± SD of 4 wells. *, ** Significantly different from the 0 hr group at \( p<0.05 \) and \( 0.01 \), respectively.

Expression of mRNAs for enzymes involved in GSH synthesis after irradiation with \( \gamma \)-rays at a dose of 50 cGy was examined. \( \gamma \)-GCS is a rate-limiting enzyme of the de novo glutathione synthesis pathway. As shown in Figure 2, the \( \gamma \)-GCS mRNA level increased soon after irradiation, peaked between 3 hr and 6 hr post-irradiation, and then declined slowly. GR is a key enzyme in the regeneration of reduced glutathione from the oxidized form. The induction of GR mRNA was also observed between 1 hr and 6 hr, post-irradiation, but the extent of induction was less than that of \( \gamma \)-GCS mRNA, and the expression after 12 hr post-irradiation was lower than that at time 0 (Data not shown).

A measurement time of 4 hr after irradiation and a dose of 50 cGy were selected for use in the subsequent...
Figure 2. Effect of doses of γ-rays on the total glutathione (GSH + GSSG) level in RAW 264.7 cells at 4 hr post-irradiation

Each point indicates the mean ± SD of 4 wells. *, ** Significantly different from the non-irradiated (0 cGy) group at p<0.05 and 0.01, respectively.

Figure 3. Effect of dose rate on the total glutathione (GSH + GSSG) level in RAW 264.7 cells at 4 hr post-irradiation

The cells were exposed to 50 cGy (total absorbed dose) at each dose rate. Each point indicates the mean ± SD of 4 wells. ** Significantly different from the non-irradiated group at p<0.01.
studies on the mechanisms responsible for the elevation of intracellular glutathione level induced by γ-ray irradiation. Inhibitors used in this study include a calcium entry blocker (NIF), tyrosine-specific kinase (GEN), a protein kinase C-specific inhibitor (CALP), a calcium chelator (EGTA), and γ-GCS inhibitor (BSO). As shown in Figure 3, BSO drastically reduced the glutathione and γ-GCS mRNA level following γ-ray irradiation (50 cGy) to a value much less than that of the non-irradiated control. Both elevations were completely blocked by GEN, CALP and NIF. The calcium channel blocker (NIF) and calcium chelator (EGTA) also effectively inhibited these elevations.

Figure 4. Changes in γ-glutamylcysteine synthetase (γ-GCS) mRNA level in RAW 264.7 cells after low-dose γ-ray irradiation at a dose of 50 cGy
Panel A: Time course of γ-GCS mRNA level in RAW 264.7 cells irradiated with γ-rays at a dose of 50 cGy. The lower panel shows the autoradiogram of the same filter rehybridized with a probe specific for GAPDH. Panel B: Results of quantification of mRNA levels by densitometric analysis of the upper autoradiogram in RAW 264.7 cells irradiated with γ-rays. Relative mRNA levels are indicated as the ratio of the γ-GCS mRNA level to the mRNA level of the housekeeping gene GAPDH.
Figure 5. Effects of various inhibitors on the elevated total glutathione (GSH + GSSG) content and γ-GCS mRNA expression induced by γ-ray irradiation at a dose of 50 cGy in RAW 264.7 cells

The RAW 264.7 cells were treated with each inhibitor for 30 min followed by exposure to 50 cGy of γ-rays. The total glutathione and γ-GCS mRNA levels in the cells were assayed at 4 hr post-irradiation. Panel A: Effects of various inhibitors on the elevated total glutathione (GSH + GSSG) level in RAW 264.7 cells irradiated with γ-rays at a dose of 50 cGy. Panel B: Effects of various inhibitors on the elevated γ-GCS mRNA level induced by γ-ray irradiation. Results of quantification of mRNA levels by densitometric analysis of the autoradiogram in RAW 264.7 cells irradiated with γ-rays.

Relative mRNA levels are indicated as the ratio of the γ-GCS mRNA level to the mRNA level of the housekeeping gene GAPDH. NIF, nifedipine (50 µM); CALP, calphostin C (50 nM); GEN, genistein (25 µM); EGTA, ethylene glyco-bis [β-aminoethyl-ether]-N,N,N',N'-tetraacetic acid (1 mM); BSO, L-buthionine-SR-sulfoximine (0.5 mM). ## Significantly different from the non-irradiated (0 cGy) group at p< 0.01. ** Significantly different from the γ-ray irradiated (50 cGy) group at p<0.01.
DISCUSSION

Glutathione is found at high concentrations in various cells, and has multiple biological roles. The reduced form (GSH) has direct or indirect roles in many biological processes, including protein and DNA synthesis, amino acid transport, activation of enzyme activities, activation of metabolism, and protection of cells from damage caused by reactive oxygen species (ROS) (17, 18). Most of the cell damage caused by ionizing radiation is mediated by ROS generated from the interaction between radiation and water molecules in cells (19-21). Intracellular GSH scavenges these ROS and protects the cells from radiation toxicity (22, 23). Several authors have suggested that GSH is involved in the resistance of many cells to radiation; the higher the level of cellular GSH, the greater the resistance to radiation. Cells with low contents of GSH are generally sensitive to radiation. High doses of radiation generate large amounts of ROS, resulting in a decrease of cellular GSH and a corresponding increase of oxidized glutathione (GSSG). Thus, it has been suggested that redox ratio (GSH/GSSG) in tissues, e.g., particularly in blood, can be used as an index of radiation-induced oxidative stress.

We have recently found that low doses (25-50 cGy) of radiation do not always cause a decrease of cellular GSH level, unlike higher doses, but in fact can increase the GSH level in mice in vivo (10). These increases were accompanied by elevated activities of GSH synthesis-related proteins following enhanced transcription of the mRNAs (11, 12).

In order to elucidate the mechanisms involved in this phenomenon, we wished to conduct experiments using a cultured cell line which has a high glutathione response to radiation. Since the high induction of intracellular glutathione after low-dose γ-ray irradiation (50 cGy) was observed in mouse macrophage-like RAW 264.7 cell, this cell-line was selected for use in this experiment.

The intracellular glutathione of RAW 264.7 cells increased soon after γ-ray irradiation (50 cGy), peaked between 3 hr and 6 hr, and returned to the non-irradiated control level at 24 hr. This transient elevation of glutathione was similar to that in tissues of mice exposed to the same dose of γ-rays (50 cGy). Concerning the dose dependency of this effect, significant elevation of the glutathione level was observed at doses of between 25 and 100 cGy at 4 hr post-irradiation. Changes in the glutathione level were also investigated at different dose rates of irradiation at a constant absorbed dose of 50 cGy. Dose-rates over 50 cGy/min significantly increased the glutathione level (p<0.05).

Further, induction of mRNAs for GSH synthesis-related proteins was examined in order to determine whether the elevation of glutathione level is a post-transcriptional event. The mRNAs for γ-GCS and GR, which are rate-limiting enzymes in the de novo pathway and the regeneration cycle for glutathione synthesis, respectively, were induced in almost the same fashion post-irradiation; both were induced soon after irradiation (50 cGy), and reached a maximum at between 3 and 6 hr post-irradiation (Figure 2). However, the maximum stimulation (fold) of γ-GCS mRNA is much higher than that of GR mRNA, suggesting that the overall increase of glutathione in the RAW 264.7 cells soon after low-dose γ-ray irradiation predominantly occurs through the operation of the de novo pathway after γ-GCS induction.

Another important aspect of this study was the assessment of the mechanisms involved in the radiation-induced elevation of intracellular glutathione. Elevation of glutathione induced by radiation may be related to the stimulation of PKC signal transduction, since the PKC pathway is considered to be involved in the expression of oncoproteins such as c-fos, c-myc, and c-jun in response to various stimuli [24-27]. It has been shown that c-fos, c-myc, c-jun and c-Ha-ras are induced by low doses of γ-rays, and PKC is commonly involved in the expression of these gene expressions [28-30].

In order to confirm that the increase of glutathione levels in RAW 264.7 cells soon after low-dose γ-ray irradiation is due to operation of the de novo glutathione synthesis pathway after transcription, the inhibitory effects of various inhibitors were examined. As shown in Figure 3, the γ-GCS inhibitor BSO reduced the both levels of glutathione and γ-GCS mRNA expression after irradiation to below that of the non-irradiated control, suggesting that the glutathione synthesis through the de novo pathway was completely blocked. GEN, a tyrosine-specific inhibitor, significantly inhibited the both increases of glutathione level and the mRNA expression induced by γ-rays, indicating that a tyrosine kinase-dependent signaling pathway is involved. Tyrosine phosphorylation is one of the early responses in the cell to various stimuli, and it was shown to be essential for cell activation in B cells (31). It is also a mandatory proximal step in the radiation-induced activation of the protein kinase C signaling pathway (32). CALP, which interacts with regulatory domain of PKC, is a specific inhibitor of PKC, and almost completely blocked the glutathione elevation and γ-GCS mRNA expression induced by radiation.

It has been well documented that Ca2+ is a signal transducer in many signaling pathways that involve the activation of PKC. NIF, a Ca2+ channel blocker, also abolished the both elevations induced by the radiation. These results suggest that the calcium channel and PKC pathways are also involved in the increase of intracellular glutathione post-irradiation with low-doses of γ-rays. The inhibitory effect of EGTA on the
elevations of glutathione and γ-GCS mRNA expression may also support this idea. The calcium channel and the PKC pathway are both involved in ROS-induced genotoxicity (33), as well as the enhanced expression of some genes induced by low-frequency magnetic fields (34-36). It remains to be determined whether different signaling pathways and mechanisms are involved in the responses to these different stimuli.

In conclusion, our results suggest that tyrosine kinase, calcium channels, and protein kinase C pathways are all involved in the low-dose radiation-induced elevation of cellular glutathione.

ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research (11680556) from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES