Role of Reactive Oxygen Species in Gallic Acid-Induced Apoptosis

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We earlier demonstrated that gallic acid (3,4,5-trihydroxybenzoic acid) induced apoptosis in promyelocytic leukemia HL-60/RG cells, which was inhibited by catalase and intracellular Ca\(^{2+}\) chelator. In this study, we further studied the involvement of reactive oxygen species (ROS) and intracellular Ca\(^{2+}\) in gallic acid-induced apoptosis. The enhancement of intracellular ROS in HL-60/RG cells was detected dose-dependently as early as 5 min after stimulation with gallic acid by using 5,6-carboxy-2',7'-dichlorofluorescin diacetate (DCFH-DA). Further studies that used various antioxidants and ROS scavengers showed that the intracellular peroxide level was well correlated with the potency to induce apoptosis and that the increased intracellular peroxides after gallic acid treatment seemed likely to result from the influx of H\(_2\)O\(_2\) derived from superoxide which were generated extracellularly. In addition, gallic acid, H\(_2\)O\(_2\), and H\(_2\)O\(_2\)-induced apoptosis was completely inhibited by pretreatment with intracellular Ca\(^{2+}\) chelator 1,2-bis(2-aminophenoxyethane)-N\(_2\)N\(_4\)N\(_6\)-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM), but increase of intracellular peroxide levels by gallic acid were suppressed only slightly. It is suggested that intracellular ROS induced by gallic acid plays an important role in eliciting an early signal in apoptosis. Especially, H\(_2\)O\(_2\) which is derived from superoxide anion generated extracellularly may increase intracellular Ca\(^{2+}\) levels or cooperate with intracellular Ca\(^{2+}\), thus resulting in apoptosis induction.

Key words gallic acid; apoptosis; reactive oxygen species; Ca\(^{2+}\) ion

Reactive oxygen species (ROS) are involved in the pathogenesis of various diseases including carcinogenesis, atherosclerosis and inflammatory disorders. Recent accumulating evidence indicates that ROS can activate signal transduction pathway, resulting in modification of gene expression as well as posttranslational modification of proteins,\(^1\) although excess generation of ROS has a harmful effect on cell function. Important steps in the signal transduction cascade such as Ca\(^{2+}\) mobilization and protein phosphorylation are regulated by physiological oxidant-antioxidant homeostasis, especially the thiol-disulfide balance.\(^2\) ROS are also known to play an important role in cell death induction and their intensity as signals decides the prevalence of apoptosis or necrosis.\(^3\) Among ROS, hydroxyl radical, hydrogen peroxide (H\(_2\)O\(_2\)), superoxide anion and lipid hydroperoxide have been shown to induce various biological responses as apoptosis, cell growth, cell adhesion, and HIV activation. In fact, H\(_2\)O\(_2\) can activate a transcription factor such as NF-\(\kappa\)B\(^5\) and AP-1,\(^5\) inhibit phosphotyrosine phosphatase activity,\(^6\) and stimulate mitogen-activated protein kinase activity\(^7\) and so on. Superoxide is found to stimulate inositol trisphosphate (IP\(_3\))-induced Ca\(^{2+}\) release\(^8\) and activate SoxR protein which positively regulates some genes involved in protection against oxidative stress.\(^9\)

However, the roles of ROS in apoptosis induction remain controversial. ROS, being highly reactive and generally non-specific, are unlikely to mediate the highly coordinated signaling pathway as common denominators. Furthermore, in anaerobic condition where ROS are not generated, cells can still undergo apoptosis.\(^10,11\) On the other hand, apoptosis in cancer cells induced by anti-cancer agents, camptothecin, etoposide, cisplatin, vinereistine, methotrexate, and adriamycin\(^12-16\) requires ROS to mediate the death signals. In addition, Fas- and transforming growth fator (TGF)-\(\beta\)-induced apoptosis are also known to be mediated by ROS.\(^17,18\) This accumulating evidence suggests that ROS may play a critical role in induction of apoptosis in some models as signaling molecules, but not a common denominator.

Gallic acid (3,4,5-trihydroxybenzoic acid), a naturally occurring plant phenol and antioxidant, induced apoptosis in the cancer cells HL-60/RG, HeLa, dRHL-84, PLC/PRF/5 and KB cells which have higher sensitivity than normal cells, such as rat primary cultured hepatocytes, macrophages, endothelial cells and fibroblasts.\(^19,20\) Studies to determine the signaling pathway leading to apoptosis in various kinds of cell lines using several enzyme inhibitors showed intracellular Ca\(^{2+}\) and ROS to be common denominators, although the death signal induced by gallic acid varied among different cell lines.\(^21\) Therefore, in this study we attempted to confirm the roles of ROS and Ca\(^{2+}\) necessary for gallic acid-induced apoptosis in order to identify the mechanism by which gallic acid elicits apoptosis in cancer cells.

MATERIALS AND METHODS

Chemicals Gallic acid (Nacalai Tesque Co. Kyoto, Japan) was recrystallized from hot water and used for the following experiment. Fetal calf serum (FCS) and RPMI 1640 were purchased from Irvine Scientific Co. (Santa Ana, CA, U.S.A.). Antibiotics (penicillin and streptomycin) were from Life Technologies Inc. (Grand Island, NY, U.S.A.). DCFH-DA (2',7'-dichrolofluorescin diacetate) was purchased from Molecular Probes (Eugene, Oregon, U.S.A. and Leiden, The Netherlands). Menadione (menadione sodium bisulfite), hypoxanthine, xanthine oxidase (xanthine oxidoreductase, Grade IV) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell Culture HL-60RG (human promyelocytic leukemia) cells were provided from the Japan Cancer Research Resource Bank. The cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 50 U/ml of penicillin and 50 \(\mu\)g/ml of streptomycin.

Fluorescent Measurement of Intracellular Peroxides HL-60RG cells (1.2 \(\times\) 10\(^6\) cells/10 ml/dish) were loaded with
5 μM DCFH-DA (dissolved in dimethylsulfoxide at 5 mM) for 30 min at 37°C. The cells were washed and suspended in 1 ml phosphate-buffered saline (PBS). Gallic acid (at a final concentration of 50 μg/ml), menadione (at a final concentration of 50 μM), hypoxanthine (at a final concentration of 1 mM), xanthine oxidase (at a final concentration of 10 mU/ml) and hydrogen peroxide (at a final concentration of 50 μM) were added just before analysis using FACSscan (Becton Dickinson). At 0, 5, 10, 20 min after addition of gallic acid or other ROS-generating agent, cell fluorescence was determined. In the study to determine the effect of inhibitors, each inhibitor was added 30 min before addition of gallic acid or the ROS-generating agent.

Assessment of Mitochondrial Membrane Potential To evaluate mitochondrial membrane potential, HL-60RG cells (1.2×10⁶ cells/10 ml/dish) were labeled with DiOC6 (40 nM in PBS) at 37°C for 15 min. After washing, gallic acid (at a final concentration of 50 μg/ml) was added to cells and then analyzed by FACSscan using carbonyl cyanide m-chlorophenyl hydrazone as a control agent.

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis HL-60RG cells (3×10⁶ cells/10 ml/dish) exposed to 50 μg/ml gallic acid, 50 μM hydrogen peroxide, 50 μM menadione, or 1 mM hypoxanthine and 5 U/ml xanthine oxidase for 3 h were collected in a tube and then washed with PBS. The cells were incubated for 10 min in 500 μl of lysis buffer (20 mM Tris–HCl pH 7.4, 10 mM EDTA, 0.2% Triton X-100) at room temperature and centrifuged at 10000 g for 10 min at 4°C. The supernatant was incubated overnight at 50°C with 100 μg/ml proteinase K. DNA was extracted with 1 vol of chloroform–phenol (1 : 1), precipitated from the aqueous phase with 1 vol of isopropanol and 500 mM NaCl at −20°C overnight and collected by centrifugation at 14000 g for 30 min at 0°C. The pellet was suspended in 70% ethanol, centrifuged at 14000 g for 10 min at 0°C, then dried under reduced pressure and incubated in 25 μl of 10 mM Tris–HCl, pH 7.5 and 1 mM EDTA for 1 h at 37°C with 1 μg/ml RNase A. Samples were heated at 65°C for 10 min and applied to agarose gel electrophoresis after addition of loading buffer. Horizontal electrophoresis was performed for 1 h at 80 V in 1.5% agarose gel with Tris–borate/EDTA electrophoresis buffer (×0.5). After treatment of gel with 0.5 μg/ml of ethidium bromide for 10 min, DNA was visualized by UV illumination.

RESULTS

ROS are known to be involved in apoptosis induction in a number of systems. We therefore measured intracellular ROS generation in HL-60RG cells after treatment with gallic acid using DCFH-DA which reacts with peroxides to emit fluorescence. Treatment of HL-60RG cells with 50 μg/ml of gallic acid caused a time-dependent increase in intracellular peroxide levels as demonstrated by an increase in fluorescence due to oxidation of 2',7'-dichlorofluorescin (DCFH) (Fig. 1). This increase in production following gallic acid treatment was dependent on the dose of gallic acid as shown in Fig. 2. As DCFH is known to rapidly react with H₂O₂ and lipid peroxides to be oxidized, we next determined the effects of several ROS on intracellular fluorescence intensity in order to determine how gallic acid elicits intracellular peroxides. H₂O₂, menadione which triggers the intramitochondrial hypergeneration of ROS, and hypoxanthine–xanthine oxidase (HX/XO) which induces superoxide extracellularly, also in-

![Diagram](image-url)
creased the fluorescence intensity in HL-60RG cells. We then examined the effects of antioxidants and radical scavengers on the enhanced intracellular peroxide levels induced by gallic acid in respect to H₂O₂, menadione and HX/XO. Gallic acid-induced peroxide production was suppressed by antioxidant N-acetyl-L-cysteine (NAC), ascorbic acid, superoxide dismutase (SOD) in the presence of catalase, and by catalase (Fig. 3), although the inhibitory effect of catalase was less than the others. In contrast, when H₂O₂-induced intracellular peroxide generation was studied, catalase and SOD/catalase suppressed the enhancement of intracellular peroxide levels to a similar degree, although antioxidant ascorbic acid and NAC failed to prevent peroxide generation. Considering the reactivity of H₂O₂ with DCFH or NAC, it was reasonable that NAC did not suppress the increase in fluorescein derived from DCF. Furthermore, menadione, which induces the per-

meability transition of mitochondria and releases superoxide, showed different responses to ROS scavengers. That is, catalase, SOD/catalase, and ascorbic acid slightly decreased intracellular peroxide generation by menadione, whereas NAC effectively suppressed it. HX/XO also increased intracellular peroxide levels and catalase, SOD, ascorbic acid, and NAC significantly inhibited intracellular peroxide formation. We reported earlier that gallic acid induces apoptosis in HL-60RG cells. Then, we studied whether menadione, HX/XO, and H₂O₂ induced apoptosis at a concentration used in this study and whether various antioxidant and radical scavengers affected apoptosis. Six hours incubation with gallic acid (50 µg/ml), HX/XO (1 mM/10 U/ml), and H₂O₂ (50 µM) induced apoptosis in HL-60RG cells, whereas menadione (50 µM) only induced morphological change and was not able to induce apoptosis even at higher concentrations of 100 and 200 µM (Fig. 4). Catalase prevented gallic acid, HX/XO, and H₂O₂-induced apoptosis completely, but not menadione-induced morphological change (Table 1), while NAC prevented gallic acid, and HX/XO-induced apoptosis and menadione-induced morphological change, but not H₂O₂-induced apoptosis. Ascorbic acid inhibited gallic acid and HX/XO-induced apoptosis, whereas it failed to inhibit H₂O₂-induced apoptosis or menadione-elicted morphological change. These results demonstrated that gallic acid and HX/XO have a similar inhibitory profile, thus suggesting that gallic acid serves as an extracellular superoxide generator.

Now that calcium is recognized as another common denominator the in signaling pathway elicited by gallic acid, we investigated the relationship between intracellular ROS and Ca²⁺ elevation. When HL-60RG cells were treated with gallic acid in the presence of intracellular Ca²⁺ chelator 1,2-bis(2-aminophenoxy)-ethane-N,N,N′,N′-tetra-acetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM), at a concentration at which gallic acid, HX/XO, and H₂O₂-induced apoptosis and menadione-induced morphological change were completely abolished (Table 1), intracellular peroxide production was slightly suppressed (Fig. 5). These results suggest that intracellular peroxide production is intimately related to intracellular Ca²⁺ elevation resulting in apoptosis induction.
Table 1. Effect of Antioxidants and Radical Scavengers on ROS-Induced Apoptosis of HL-60RG Cells

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Symbols indicate that the agent did not inhibit (×) or did inhibit (O) apoptosis (as determined by microscopic observation) or induce morphological change (△).

Syringic acid, protocatechuic acid, and ethyl gallate which possess similar structures as gallic acid, but do not induce apoptosis, suppressed ROS-mediated signal transduction by cytokines or hormones. Excess ROS, however, cause damage to cells by oxidizing lipids in cell membrane or by attacking DNA directly.

We previously demonstrated that ROS and intracellular Ca²⁺ served as common stimuli in gallic acid-induced apoptosis. Therefore, we first studied the relationship between intracellular ROS levels and the potency to induce apoptosis in gallic acid-induced apoptosis. We found that gallic acid elevated intracellular peroxide levels in HL-60RG cells from a few minutes after gallic acid treatment and that these elevated levels were effectively suppressed by treatment of cells with catalase, SOD/catalase, or ascorbic acid, which protected the cells from gallic acid-induced apoptosis. This indicated implicated intracellular ROS in the induction of apoptosis. Furthermore, NAC, which is a powerful scavenger of hydroxyl radical like other thiols and reacts slowly with H₂O₂, but not with superoxide, effectively suppressed gallic acid-induced intracellular peroxide generation in HL-60RG cells. That is, gallic acid was likely to generate H₂O₂ or hydroxyl radical in cells.

In the case of H₂O₂-induced apoptosis, catalase effectively inhibited apoptosis and reduced intracellular peroxide levels. However, ascorbic acid did not scavenge H₂O₂ and NAC appeared not to be enough to scavenge H₂O₂ at the concentration used in this study. In addition, menadione induced morphological change but not apoptosis in HL-60RG cells, although intracellular peroxide was maintained at high levels. HX/XO-induced oxidative stress showed a similar inhibitory profile to gallic acid. Taken together, gallic acid seems to generate superoxide extracellularly to transmit death signals as HX/XO does. Furthermore, considering the membrane permeability of catalase, SOD and antioxidants in addition to various ROS, the increased intracellular peroxides seem likely to result from the influx of H₂O₂ derived from superoxide which was generated by auto-oxidation of gallic acid.

Recent multiple lines of evidence indicate that mitochondrial alterations are closely linked to apoptosis induction and that nuclear apoptosis is preceded by the disruption of the mitochondrial transmembrane potential. Many agents are known to induce mitochondrial permeability transition, which can induce release of cytochrome c that activates caspase 9, glutathione depletion, ROS hyperproduction and intracellular Ca²⁺ elevation, finally resulting in DNA fragmentation and apoptosis induction. We therefore examined the effect of gallic acid on mitochondrial transmembrane potential using 3,3',dihexyloxacarbocyanine iodide. When the potential of HL-60RG cells after treatment with gallic acid was measured, change of potential was not observed, thus suggesting that gallic acid did not influence mitochondria to generate ROS or apoptotic signals. These results coincided with recent studies that the collapse of the inner mitochondrial transmembrane potential is not required for induction of apoptosis in HL-60 cells, and that induction of mitochondrial permeability transition and cytochrome C release in the absence of caspase activation is insufficient for effective apoptosis of HL-60 cells. This kind of evidence supported our results that menadione, which indeed induces mitochondrial permeability transition, did not cause apoptosis and that gallic acid did not induce mitochondrial permeability transition, but elicited apoptosis.

Accumulating biochemical evidence indicates that H₂O₂ stimulates some signaling pathways, although the concentration of H₂O₂ used in those studies was considerably high. For example, H₂O₂ increases the synthesis of cyclooxygenase- and lipooxygenase-derived arachidonic acid metabolites through the activation of phospholipase A₂, the levels of both diacylglycerol and IP₃ through enhanced hydrolysis of phosphatidylinositol 4, 5-bisphosphate by phospholipase C, the level of phosphatidic acid through endothelial cell phospholipase D activation, protein tyrosine phosphorylation, and agonist-sensitive Ca²⁺-influx. In addition, both superoxide and H₂O₂ are found to inhibit ATP-dependent Ca²⁺ pump and to stimulate Ca²⁺ release from inositol 1,4,5-trisphosphate-sensitive store.

In the present study, ROS- and gallic acid-induced apoptosis were found to be inhibited by pretreatment intracellular Ca²⁺ chelator BAPTA-AM effectively. These results prompted us to speculate that there are two possible signaling pathways in gallic acid-induced apoptosis. The first is that intracellular...
ROS generation, even at a low concentration, is followed by Ca\(^{2+}\) elevation in gallic acid-induced apoptosis. The second is that ROS and Ca\(^{2+}\) elevation are induced independently, but both are required for apoptosis induction. However, the mechanism by which gallic acid increases intracellular Ca\(^{2+}\) levels is still obscure. Further studies on the mechanism by which gallic acid induces apoptosis are currently in progress and might offer new insight into the role of ROS and Ca\(^{2+}\) in the signal transduction of cell death.

REFERENCES