Antioxidant Regulation of Protein Kinase C in Cancer Prevention

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ABSTRACT Besides scavenging free radicals, antioxidants inhibit signaling enzymes such as protein kinase C (PKC) that play a crucial role in tumor promotion. By having different oxidation susceptible regions, PKC can respond to both oxidant tumor promoters and cancer-preventive antioxidants to elicit opposite cellular responses. Oxidant tumor promoters activate PKC by reacting with zinc-thiolates present within the regulatory domain. In contrast, the oxidized forms of some cancer-preventive agents, such as polyphenolics (ellagic acid, 4-hydroxytamoxifen and curcumin) and selenocompounds, can inactivate PKC by oxidizing the vicinal thiols present within the catalytic domain. This brings an efficient counteractive mechanism to block the signal transduction induced by tumor promoters at the first step itself. Because prostate cancer prevention clinical trials in large human population are under way, we have focused more on understanding the cancer-preventive mechanism of selenium. Methylselenol, the postulated cancer-preventive metabolite, has no direct effect on PKC activity. However, methylseleninic acid, locally generated by the reaction of membrane methylselenol with PKC-bound tumor-promoting fatty acid hydroperoxides, selectively inactivates PKC. This mechanism clarifies how the volatile methylselenol that is present in a low concentration induces the inactivation of PKC selectively in the promoting precancer cells. Selenoprotein thioredoxin reductase reverses selenium-induced inactivation of PKC, suggesting that selenoproteins may serve as a safeguard against the toxicity induced by selenometabolites. Moreover, this also explains how a resistance to selenium develops in advanced malignant cells. The redox-mediated inactivation of PKC may, at least in part, be responsible for the antioxidant-induced inhibition of tumor promotion and cell growth, as well as for the induction of cell death. J. Nutr. 132: 3819S–3823S, 2002.

KEY WORDS: antioxidants • selenium • cancer prevention • protein kinase C • thioredoxin reductase

Both epidemiologic and experimental data suggest that a variety of dietary antioxidants can reduce the risk of cancer in humans (1–3). Given the causative role of oxidants in carcinogenesis (4,5), dietary antioxidants are important in cancer prevention. The conventional view held for a long time is that antioxidants act by scavenging free radicals. Although these actions of antioxidants are certainly important in preventing promutagenic DNA damage caused by oxidants, other actions of antioxidants, particularly those influencing cell signaling mechanisms, have also recently come into light (6,7). Antioxidants are believed to induce their own effects on cell signaling in the precancer cells to decrease tumor promotion, a critical stage in carcinogenesis (5). Nevertheless, the exact mechanism by which these additional actions of antioxidants inhibiting tumorigenesis is not known.

Although antioxidants can overall decrease the tumorigenesis process, they are not effective in preventing cancer in some cases. In certain cases, they may even enhance carcinogenesis (3). Thus, understanding molecular mechanisms by which dietary antioxidants inhibit carcinogenesis is essential. It will help us understand the conditions that optimize their cancer-preventive action and eventually lead to the development of effective cancer-preventive strategies.

Current issues in understanding antitumor-promoting actions of antioxidants

Although a variety of enzymes involved in signal transduction pathways are indirectly affected in the cells treated with various antioxidants, it is important to know the specific molecular targets that are directly influenced by antioxidants (8,9). Furthermore, it is not clear whether these direct effects are caused by a physical binding of antioxidants to the target molecule or by redox modifications of the target by antioxidant molecules. It is currently believed that cancer-preventive actions of antioxidants are mediated by inhibition of critical cellular targets such as protein kinases, growth factor receptors or other enzymes involved in the cellular signaling (6,7,9,10). Although these are very relevant targets for cancer prevention, these critical enzymes are also present in the normal cells, and their inhibition is expected to produce severe toxicity to the host. Therefore, it is important to explain how antioxidants interrupt cell signaling selectively in the precancer cells.
but not in the normal cells. In addition, the concentrations of antioxidants needed to induce tumor cell growth inhibition or cell death in cell culture are often severalfold higher than the concentrations of the free compound that are reached in the tissues by dietary supplementation. These in vitro effects of antioxidants are very relevant for their antitumor-promoting activity (9), but it is important to explain how such effects occur at the bioavailable low concentrations of antioxidants.

**Protein kinase C as a target for both tumor promoters and antitumor promoters**

Protein kinase C (PKC) represents a family of >11 phospholipid-dependent serine/threonine kinases that are involved in a variety of pathways that regulate cell growth, death and stress responsiveness (5,11). The isoforms are divided into three categories (11). Conventional PKC isoenzymes (5,11). The isoforms are divided into a variety of pathways that regulate cell growth, death and stress responsiveness (5,11). The isoforms are divided into three categories (11). Conventional PKC isoenzymes (α, β and γ) are Ca2⁺ dependent and are stimulated by second messenger diacylglycerol. Novel PKC isoenzymes (δ and ε) are Ca2⁺ independent but are also diacylglycerol stimulatable. A typical PKC isoenzyme (δ and A) require neither Ca2⁺ nor diacylglycerol for optimal activity. Phorbol ester–type tumor promoters activate PKC by directly binding to and substituting for diacylglycerol (11). PKC regulates tumor promotion and cell growth by inducing activation of transcriptional factors such as activator protein-1 and nuclear factor-κB, and by increasing the expression of key enzymes such as ornithine decarboxylase, inducible nitric oxide synthase and cyclooxygenase-2 (12,13).

PKC has unique structural aspects that render it susceptible to activation by oxidant tumor promoters such as H₂O₂, peroxide and tobacco-related tumor promoters (14–16). Selective oxidative modification of the regulatory domain results in Ca2⁺/lipid-independent activation, whereas selective oxidative modification of the kinase domain results in inactivation (5,17). The regulatory domain contains 12 cysteine residues that coordinate the binding of four zinc atoms: the zinc-thiolate structure is required for binding of phorbol ester and diacylglycerol (18). This positively charged zinc-thiolate is more susceptible to tumor-promoting oxidants (5,17). Thus, phorbol esters activate the enzyme by binding to the structure supported by zinc-fingers, whereas oxidants directly induce a similar effect by reacting with zinc-thiolates and inducing the collapse of the zinc-fingers (Fig. 1). In both cases, changes occurring in the regulatory domain relieve its autoinhibitory effect caused by the interaction of its pseudosubstrate region with the protein substrate-binding region of the catalytic domain (C4).

Although our original hypothesis is that antioxidants block oxidant effects on PKC, we have found that they have a direct inhibitory effect on PKC. Some cancer-preventive agents, such as polyphenolics (curcumin, 4-hydroxy-tamoxifen and ellagic acid) and selenocompounds in their oxidized state, can inactivate PKC by oxidizing the vicinal thiols present within the catalytic domain (19–23). Whether these types of oxidized metabolites are formed in the body is not known. Nevertheless, there has been a significant progress in identifying the type of metabolites formed from the dietary selenocompounds (24,25). In addition, prostate cancer prevention clinical trials in large human population are under way (26). Therefore, we have focused on understanding the inactivation mechanism of PKC by selenium with respect to its specificity to PKC isoenzymes, selectivity to precancer cells and relevance to its antitumor-promoting action.

**Issues related to cancer-preventive mechanism of selenium**

Experimental studies in animals indicate that selenium supplementation at levels (1 to 3 mg/kg) above the dietary requirement (0.1 mg/kg) can prevent tumorigenesis at various sites, including breast and skin (24–27–29). Landmark clinical trials carried out by Clark et al. (30) suggested that supplemental selenium may reduce the incidence of and death from prostate, lung and colorectal cancers but not of skin. Therefore, it is imperative to know why selenium prevents cancers in some cases while it fails to prevent cancer in other cases.

Selenocompounds, such as selenite, selenomethionine and Se-methylselenocysteine, ultimately generate selenide, which is incorporated into the selenoproteins glutathione peroxidase (GPX) and thioredoxin reductase (TR) by a specialized mechanism (24,25). Furthermore, selenide is sequentially methylated to methylselenol, dimethylselenide and trimethylselenonium (24). Both methylselenol and trimethylselenonium are excreted through urine, while dimethylselenide is exhaled (Fig. 2). Initially, it was hypothesized that the chemopreventive effect of selenium might be mediated by selenoproteins such as GPX to remove tumor-promoting peroxides. The activities of known selenoproteins seem to be saturated in animals at a much lower dietary selenium level (0.1 mg/kg) than that required for cancer prevention (1 to 3 mg/kg). Due to this limitation, selenometabolite methylselenol has been implicated in inducing the cancer-preventive actions of selenium (24,25). Other important cancer-preventive selenocompounds such as 1,4-phenylenedis(methylene) selenocyanate are also believed to mediate cancer-preventive actions after conversion to selenol metabolite (31). Although selenite, selenomethionine, methylselenol and other redox-active selenometabolites can induce certain cancer-preventive actions in cell culture, they are present in very low concentrations in plasma and tissues (27,32–34). Despite these in vitro cellular actions of selenometabolites are very relevant to understand the cancer-preventive mechanisms, it is important to determine

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**Note:** Abbreviations used: GPX, glutathione peroxidase; PKC, protein kinase C; TR, thioredoxin reductase.
PKC and the homologous cysteine residues are not present in other protein kinases (23). Some of these cysteine residues are missing in the Ca$^{2+}$-independent PKC isoenzymes. In the catalytic domain of δ, ε and ζ isoenzymes, one conserved cysteine (residue 569 in α-isoenzyme) is absent, whereas in ε isoenzyme, an additional cysteine (residue 383 in α-isoenzyme) is also absent (23). Thus, there is a structural basis for the differential sensitivity of various PKC isoenzymes to react with selenium. Other redox-sensitive enzymes such as protein kinase A, phosphorylase kinase, phosphorylase phosphatase and protein phosphatase 2A were not sensitive to methylseleninic acid, and an appreciable inactivation was observed only at high concentration (>25 μM) of methylseleninic acid.

If seleninic acid is formed elsewhere in the cell and reacts with PKC, it may not be very specific in its reactivity with PKC. We have determined whether a local formation of methylseleninic acid gives more specificity to PKC. Unsaturated fatty acids and phospholipids and their peroxides bind to and activate PKC (35).

These hydroperoxides cannot directly modify sulfhydryls in PKC. When PKC was preincubated with arachidonic acid hydroperoxide, methylselenol inactivated PKC at a concentration as low as 50 nM (Fig. 4). Such a low concentration of exogenous methylseleninic acid was not effective in inactivating PKC. However, when methylselenol or other lipophilic selenols reacts with the PKC-bound fatty acid hydroperoxides in the membrane, the locally generated seleninic acid reacts with PKC sulfhydryls present within the vicinity. Because the cysteine residues present within the catalytic domain do not coordinate zinc binding, they readily react with seleninic acid, leading to the loss of kinase activity. However, if seleninic acid is formed at higher concentrations, it also reacts with the zinc-thiolates present in the regulatory domain. Thus, methylselenol not only blocks the action of tumor-promoting hydroperoxide to activate PKC but also induces the inactivation of the kinase. Furthermore, methylselenol, a volatile metabolite that is not readily retained in the cell, is converted to nonvolatile methylseleninic acid by reacting with tumor-promoting hydroperoxide and is retained in the cell. This mechanism explains the selectivity of methylselenol action to promotable precancer cells. After reacting with thiolis, methylseleninic acid regenerates as methylselenol and can

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**FIGURE 2** Selenium metabolism and excretion

Whether these actions occur in vivo at bioavailable concentrations of selenium, as well as how they are related to the actions of selenoproteins.

**Inactivation of PKC isoenzymes by methylseleninic acid, oxidized metabolite of methylselenol**

Methylselenol, the postulated cancer-preventive metabolite, has no direct effect on PKC activity. However, its oxidized metabolite methylseleninic acid inactivates PKC. As shown in Figure 3, methylseleninic acid inactivated Ca$^{2+}$-dependent enzymes at lower concentrations (IC$_{50}$ = 500 nM) and PKCδ and PKCe at higher concentrations. Clearly, PKCζ is the least susceptible isoenzyme tested. A requirement for a cluster of four cysteine residues to readily reduce seleninic acid (23) is creating specificity for the reaction of methylseleninic acid with PKC. The catalytic domain of PKC (Ca$^{2+}$-dependent isoenzymes), besides possessing the cysteine residue conserved among other protein kinases, has three additional conserved cysteine residues. These cysteines are very unique to PKC and the homologous cysteine residues are not present in other protein kinases (23). Some of these cysteine residues are missing in the Ca$^{2+}$-independent PKC isoenzymes. In the catalytic domain of δ, ε and ζ isoenzymes, one conserved cysteine (residue 569 in α-isoenzyme) is absent, whereas in ε isoenzyme, an additional cysteine (residue 383 in α-isoenzyme) is also absent (23). Thus, there is a structural basis for the differential sensitivity of various PKC isoenzymes to react with selenium. Other redox-sensitive enzymes such as protein kinase A, phosphorylase kinase, phosphorylase phosphatase and protein phosphatase 2A were not sensitive to methylseleninic acid, and an appreciable inactivation was observed only at high concentration (>25 μM) of methylseleninic acid.

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**FIGURE 3** Differential sensitivity of various PKC isoenzymes to methylseleninic acid. Rat brain PKC isoenzymes α and β were isolated by Ca$^{2+}$-dependent hydrophobic chromatography (37). PKC δ and ε were purified from rat brain, while PKCζ was purified from rat kidney. Desalted PKC isoenzymes free from thiol agents were preincubated with the indicated concentrations of methylseleninic acid in the wells of multiwell plate for 5 min. Then PKC activity was determined using PKCe pseudosubstrate peptide.

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**FIGURE 4** Inactivation of PKC by methylselenol in the presence of arachidonate hydroperoxide. PKC (mixture of α, β, and γ isoenzymes) was incubated with or without arachidonate hydroperoxide (25 μM) for 5 min. Then treated with the indicated concentrations of methylselenol (prepared by mixing methylselenenic acid and dithiothreitol 1:2 mol/mol). After incubation at 30°C for 5 min, the residual PKC activity was determined.
Repeat this action by reacting with hydroperoxide. Thus, a limited amount of methylselenol can oxidize many molecules of PKC by functioning as a redox catalyst.

**Reversal of selenium-induced PKC redox modification by selenoprotein thioredoxin reductase**

A calf thymus TR system (purified TR, thioredoxin, NADPH) regenerated both the kinase activity and phorbol ester binding for PKC, which was inactivated by sulfhydryl oxidation induced by redox-active selenocompounds (36). Unexpectedly, TR induced this reduction to an appreciable extent even in the absence of thioredoxin (36). TR also regenerated the phorbol ester binding for the oxidized recombinant fragment of PKC having two zinc-fingers. Furthermore, modified TR, in which selenocysteine was either selectively alkylated or removed by carboxypeptidase treatment, was ineffective. Similarly, *Escherichia coli* TR, which is not a selenoprotein, was not effective. Although PKC has no homology to thioredoxin, it has four zinc-fingers with the thioredoxin-type redox motif (C-X3-C) and a flexible protein-binding region, enabling it to directly bind and react with the seleno-sulfur redox center present in TR. If TR does not readily reverse this modification, then PKC is irreversibly inactivated by a collapse of zinc-fingers. Thus, PKC is not only a specific target for selenometabolites but also an appropriate target for selenoprotein TR and thus serves as a point of integration for both modes of actions of selenium.

**PKC and interrelationship between selenometabolites and selenoproteins.** Although the reactions of selenometabolites with PKC can induce antitumor-promoting actions, the reversal of these reactions by TR can prevent the toxic actions of selenometabolites in normal cells. During oxidative stress TR is known to be inactivated by the loss of selenium from the enzyme (37), or its activity is diminished by the limited supply of reducing equivalents (NADPH). This compromise in the action of TR could allow the toxic action of selenometabolites leading to the inactivation of PKC. This may provide selectivity in the action of selenometabolites to precancer cells versus normal cells. It is also possible that in some advanced tumor cells, which are resistant to selenium, an induction of TR, along with the cellular ability to generate sufficient amounts of reducing equivalents, can give them resistance to selenium toxicity. Previous studies have shown that selenide reacts with oxygen and induces the generation of H$_2$O$_2$ (38). The enzyme that protects cells from this toxicity is a selenoprotein, GPX. Furthermore, selenoprotein TR either directly or indirectly reverses selenium-induced sulfhydryl oxidation in PKC. Therefore, selenoproteins serve as a safeguard against the toxicity induced by selenometabolites but also protect cells from global oxidative stress.

**Significance of PKC inhibition to cancer-preventive actions of selenium**

**Inhibition of tumor promotion and cell growth.** Selenium has been shown to inhibit tumor promotion (9,39). Previous studies showed that an inhibition or downregulation of PKC abolished the phorbol ester–induced induction of ornithine decarboxylase (39). Because the overexpression of ornithine decarboxylase is associated with tumorigenesis (40), selenocompounds, by blocking the induction of ornithine decarboxylase and other genes via interrupting PKC function, may elicit in part their cancer-preventive action (39). Selenium-induced inhibition of PKC may also play a role in the selenium-mediated inhibition of activator protein-1 and nuclear factor–κB transactivation in intact cells. Because PKC is an important enzyme in the induction of inducible nitric oxide synthase, cyclooxygenase-2 and other enzymes involved in tumor promotion (12,13), inhibition of PKC by selenium may have significant role in preventing the induction of these enzymes. Selenium-induced inhibition of PKC has been shown to decrease cdk2 kinase activity and subsequent arrest of tumor cell growth (41).

**Induction of apoptosis.** Various selenocompounds were shown to induce apoptosis (42). Inhibition and or inactivation of PKC induced by selenium may have a role in inducing apoptosis. Moreover, various commonly used PKC inhibitors such as calphostin C, hypericin, chelerythrine and staurosporine induce apoptosis, which further suggests that the inactivation or inhibition of PKC triggers apoptosis (43). Inhibition of PKC by its inhibitors induces apoptosis via the generation of ceramide (44). Selenium-induced inactivation of PKC in prostatic carcinoma cells also leads to an elevation of ceramide and induction of apoptosis (45). PKC was shown to act as a negative modulator for sphingomyelinase and inhibits its activity (44). Therefore, an inhibition of PKC activity leads to an activation of sphingomyelinase and an increased generation of ceramide (44). Ceramide can increase the mitochondrial generation of reactive oxygen species and increase mitochondrial transition permeability (46). Although this can be prevented by Bcl-2, its antiapoptotic function is suppressed by a lack of phosphorylations mediated by PKCa and mitogen-activated protein kinases (47). Then the ceramide-induced changes in mitochondria leads to the release of cytochrome c into cytosol, where it induces the activation of caspase-3, a key protease involved in inducing apoptotic events (48). Caspase-3 activates PKCζ via a limited proteolysis (49). Furthermore, ceramide activates PKCζ and c-Jun N-terminal kinase, which further helps in executing apoptosis. PKC isoenzymes, particularly PKCα and PKCB, are better suited for inactivation by selenocompounds to trigger early events in apoptosis, and the PKCζ and PKCδ are relatively less susceptible for this inactivation and may facilitate the later events in apoptosis. Thus, differential susceptibility of PKC isoenzymes to selenium is well suited for inducing apoptosis-related events.

Oxidant tumor promoters are capable of inducing cell death in normal cells (50). However, some premalignant or malignant cells escape cell death inducible by oxidants, and thus accumulate mutations, which ultimately leads to tumor promotion or progression (50). Low concentrations of selenol-retained and amplified by peroxides, blocks the escape of tumor cells from death and "resores" cell death. The concept of selenium “restoring” cell death is different from selenium per se inducing cell death. Selenium per se induces cell death only at high concentration by a global toxic mechanism, which affects both normal and cancer cells.

**Summary**

Oxidized forms of antioxidants induce oxidation of PKC, the same cellular target that tumor promoters act on, but induce its inactivation. This may bring an efficient counteractive mechanism to block signal transduction induced by tumor promoters. Methylselenol, the postulated cancer-preventive metabolite, reacts with PKC-bound tumor-promoting fatty acid hydroperoxide, and thus the locally generated methylseleninic acid causes specific inactivation of PKC in the precancer cells. TR, a selenoprotein, can reverse this antitu-umor-promoting action of selenium. This suggests that an interesting interrelationship exists between the actions of sel-
enorme them  and selenoproteins in regulating PKC. The inactivation of PKC may, at least in part, be responsible for the antioxidant-induced inhibition of tumor promotion and cell growth, as well as for the induction of cell death.

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