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Protease Inhibitors Interfere with the Necessary Factors of Carcinogenesis

by Walter Troll*

Many tumor promoters are inflammatory agents that stimulate the formation of oxygen radicals ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) in phagocytic neutrophils. The neutrophils use the oxygen radicals to kill bacteria, which are recognized by the cell membrane of phagocytic cells causing a signal to mount the oxygen response. The tumor promoter isolated from croton oil, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), mimics the signal, causing an oxygen radical release that is intended to kill bacteria; instead, it injures cells in the host. Oxygen radicals cause single strand breaks in DNA and modify DNA bases. These damaging reactions appear to be related to tumor promotion, as three types of chemopreventive agents, retinoids, onion oil, and protease inhibitors, suppress the induction of oxygen radicals in phagocytic neutrophils and suppress tumor promotion in skin cancer in mice. Protease inhibitors also suppress breast and colon cancers in mice. Protease inhibitors capable of inhibiting chymotrypsin show a greater suppression of the oxygen effect and are better suppressors of tumor promotion. In addition, oxygen radicals may be one of the many agents that cause activation of oncogenes. Since retinoids and protease inhibitors suppress the expression of the *ras* oncogene in NIH 3T3 cells, NIH 3T3 cells may serve as a relatively facile model for finding and measuring chemopreventive agents that interfere with the carcinogenic process.

Frequently, the development of new concepts regarding the mechanisms of biological phenomena, such as the multiple stages of carcinogenesis, requires the cooperative interaction of several individuals who look at data from different perspectives. An example is the finding that tumor promotion by inflammatory agents is due in part to a false signal acting on neutrophils to yield oxygen radicals. This finding was due to Norton Nelson's foresight and leadership in bringing together Bernard Goldstein, an eminent hematologist, Gisela Witz, an organic chemist who prepared and studied tumor promoters in Benjamin Van Duuren's lab, and myself, a biochemist interested in suppressing the action of tumor promoters. Goldstein was experienced in measuring oxygen radicals and hydrogen peroxide in human neutrophils caused by the action of invading bacteria in perturbing the cell membrane. Witz's experience with the inflammatory action of tumor promoters led to the substitution of the bacterial challenge with the pure tumor promoter TPA. TPA caused the formation of $\cdot O_2^-$ and H_2O_2, which interact with human and animal phagocytic neutrophils (*1,2*). The role of oxygen radicals in tumor promotion received some support from the observation that agents known to suppress tumor promotion are also effective inhibitors of the formation of oxygen radicals. These include the anti-inflammatory hormone dexamethasone, onion oil components blocking lipoxygenase, retinoids, and protease inhibitors (*3-5*).

Protease inhibitors were a novel entry into the group of agents that suppress the oxygen response of phagocytes. We noted the suppression of H_2O_2 in fertilized sea urchin eggs by soybean and other trypsin inhibitors and the resulting polyspermy. Fertilization by the first successful sperm induces the oxidation response, which results in the excretion of H_2O_2 that kills excess sperm. Protease inhibitors suppress the formation of H_2O_2, permitting the excess sperm to survive and causing polyspermy. Catalase, the enzyme that specifically destroys H_2O_2, also causes polyspermy. Protease inhibitors suppress the induction of H_2O_2 in the sea urchin egg (*6,7*).

Protease inhibitors suppress H_2O_2 formation induced by TPA in phagocytic neutrophils. The inhibitors of chymotrypsin appeared to be more effective than those of trypsin in suppressing oxygen radical and hydrogen peroxide formation by human neutrophils. Thus the Bowman-Birk soybean inhibitor, which is a more powerful inhibitor of chymotrypsin than the Kunitz soybean inhibitor, showed a 7-fold greater suppression of $\cdot O_2^-$ production by TPA in neutrophils than the Kunitz inhibitor (*8*). In a more extended experiment, studying H_2O_2 formation by TPA-induced neutrophils, H_2O_2 formation was increasingly blocked by a series of protease inhibitors depending on their potency to inhibit chymotrypsin. The most powerful inhibitor is the potato inhibitor 1 (PT1), which exclusively inhibits chymotrypsin.

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The order of activity of suppression of H_2O_2 formation is shown in Figure 1 and Table 1, taken from the recent publication of Frenkel et al. (*9*). We see that the PT1, which only forms a tight complex with chymotrypsin and is without activity in blocking trypsin at a 10 μM concentration, blocks hydrogen peroxide formation by 88%. The mechanism of this almost total suppression of the oxygen burst by this chymotrypsin inhibitor remains to be elucidated. It is not due to the inhibition of superoxide dismutase (SOD) (*10*), the enzyme which converts $^{\bullet}O_2^-$ to H_2O_2, since it is not overcome by adding an excess of this enzyme (Table 1). PT1, an exclusive chymotrypsin inhibitor, shows sharply ascending curves of H_2O_2 suppression at levels of 1 to 10 μM. The potato inhibitor 2 (PT2), which has prosthetic groups inhibiting both trypsin and chymotrypsin, suppressed H_2O_2 to a lesser extent (39% at 10 μM versus 88% by PT1), and the suppression plateaus at higher concentrations only reached 70% suppression at 40 μM (Fig. 1, Table 1). Moreover, the chymotrypsin inhibitor fraction isolated from PT2 is a more effective suppressor of H_2O_2 formation than PT2, containing a trypsin inhibition capacity as well (Fig. 2). This unusual property of the chymotrypsin inhibitor may be responsible for its more effective action in blocking *in vivo* and *in vitro* tumor promotion. Thus, the synthetic chymotrypsin inhibitor, tosyl-phenylalanine-chloromethyl ketone, was more effective than the analogous trypsin inhibitor, tosyl-L-lysine chloromethyl ketone, in blocking tumor promotion (*11*). The chymotrypsin prosthetic group of the Bowman-Birk soybean inhibitor, which is similar to the PT2 trypsin and chymotrypsin combining groups, was identified as the important constituent responsible for suppressing neoplastic transformation caused by ionizing radiation in C3H/10T½ cells (*12*). Indeed, Yavelow et al. have identified a chymotrypsin-type enzyme in the cell membrane of 10T½ cells, which may act as a receptor for the localization of protease inhibitors with inhibitory properties to chymotrypsin (*13*). The chymotrypsin inhibitor PT1 also inhibits radiation induced neoplastic transformation of C3H/10T½ cells (*14*).

On the other hand, trypsin inhibitors also have anticarcinogenic properties, as shown by the observation that 6-amino caproic acid, a trypsin and plasminogen activator inhibitor without any inhibitory action against chymotrypsin, suppresses chemically induced colon cancer in mice (*15*). This inhibitor, in common with all protease inhibitors, will limit the digestion of proteins to amino acids, which decreases the availability of amino acids to the growing cancer cell (*16*). This simple compound, which is closely related to the nutritionally essen-

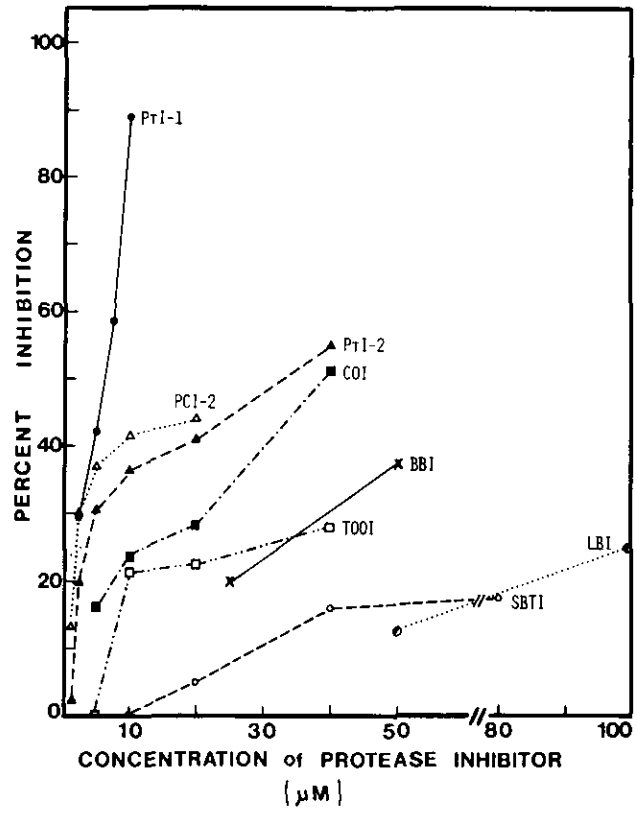

FIGURE 1. Protease inhibitor-induced decrease of H_2O_2 formation by TPA-activated human PMNs. PMNs (7.5–8.5 × 10^4) were treated with various concentrations (1.25–100 μM) of protease inhibitors just prior to activation with TPA (25 nM), incubated with phenol red and horseradish peroxidase at 37°C for 30 min, and absorbance was determined at 590 nm. Protease inhibitors used were: PtI-1 (●---●), PtI-2 (▲---▲), PCI (△ · · · △), COI (■ · · ·■), TOOI (□ · · · □), BBI (X—X), LBI (◐ · · · ◐) and SBTI (○---○). Representative experiments (*9*).

Table 1. Percentage inhibition of H_2O_2 formation by TPA-activated PMNs caused by protease inhibitors (*9*).[a,b]

Concentration of PIs	PTI-1[c]	PTI-2[c]	PCI-2[c]	COI[c]	TOOI[c]	BBI[c]	SBTI[c]
1.25	—	2.5 (1)	13.7 (1)	—	—	—	—
2.5	29.5 (1)	20.0 (1)	31.4 ± 1.0(2)	—	—	—	—
5.0	40.5 ± 1.0(2)	32.0 ± 1.0(2)	37.9 ± 0.7(4)	16.9 (1)	0.4 (1)	—	—
7.5	58.6 (1)	—	—	—	—	—	—
10	87.5 ± 1.0(2)	37.6 ± 1.3(4)	42.1 ± 0.5(2)	23.6 ± 0.1(2)	21.7 (1)	—	0.3 (1)
20	—	47.7 ± 4.7(2)	44.2 ± 0.1(2)	29.7 ± 5.9(3)	19.5 ± 1.7(2)	—	7.0 ± 1.3(2)
25	—	—	—	—	—	22.5 ± 1.8(2)	—
40	—	63.8 ± 6.4(2)	—	40.3 ± 8.0(2)	26.8 (1)	—	13.9 ± 2.7(3)
50	—	—	—	—	—	37.8 (1)	—
80	—	—	—	—	—	—	17.2 (1)

[a]Incubation at 37°C for 30 min.

[b]Numbers in parentheses show how many PMN concentrations were assayed.

[c]Abbreviations: PTI-1, potato inhibitor 1; PTI-2, potato inhibitor 2; PCI-2, chymotrypsin-inhibitory fragment of PTI-2; COI, chicken ovoinhibitor; TOOI, turkey ovomucoid ovoinhibitor; BBI, Bowman-Birk inhibitor from soybeans; SBTI, soybean (Kunitz) trypsin inhibitor.

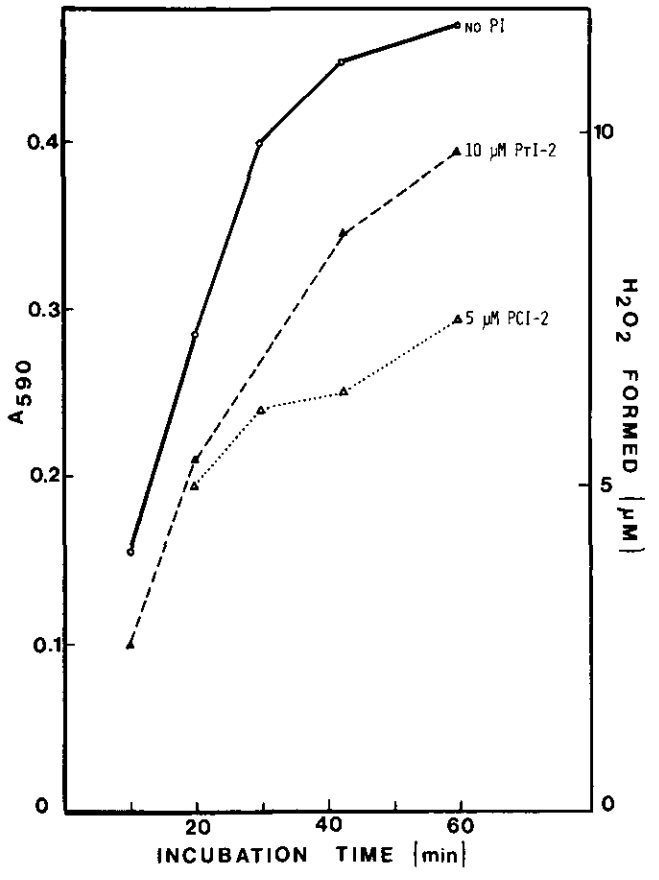

FIGURE 2. Time course of H_2O_2 formation by TPA-activated PMNs in the absence (O---O; no PI) or presence of protease inhibitors (representative experiments). PMNs (8×10^4) were treated with 5 μM PCI-2 (△ · · · △) or 10 μM PtI-2 (▲---▲) just before addition of TPA (25 nM). Samples were incubated at 37°C for 10 to 60 min and absorbance was determined at 590 nm (*9*)

tial amino acid lysine, does not block H_2O_2 formation from neutrophils but rather appears to inhibit the induction of the DNA polymerase α, an important enzyme for amplification of virus-modified DNA (*17*). Garte et al. have recently shown that 6-amino caproic acid, α_1-trypsin inhibitor and leupeptin suppress the expression of *ras* oncogene modification of NIH 3T3 cells (*18*).

Thus, there appear to be multiple mechanisms by which protease inhibitors and other chemopreventive agents interfere with the development of cancer. The role of oxygen radicals in relation to oncogene activation of expression of oncogenes needs to be investigated. The recent work by Sawey et al. showed that ionizing radiation that causes tumors on rat skin also induces specific oncogenes (*19*). As ionizing radiation causes the formation of oxygen radicals, they may be responsible for the activation of oncogenes (*19*). The mechanisms of the chemopreventive action of retinoids, including their blocking of tumor promotion and experimental breast cancer, may also be related to their antioxidant properties, as shown by their blocking of oxygen radical formation in TPA-induced neutrophils (*1*, *2*). It is of interest that all *trans*-retinoic acid has recently been shown to block *ras* oncogene expression in NIH 3T3 cells (S. J. Garte, personal communication).

Thus, protease inhibitors and retinoids, chemopreventive agents of different types, suppress oncogene expression. This suggests that the technique of studying oncogene expression may serve as a facile assay for identifying and measuring chemopreventive agents. It is less costly and requires less time than the conventional methods of causing animal cancer by a carcinogen and preventing it by presumptive chemopreventive agents applied to the tissue or in the food of the experimental animal. The suppression of oncogene expression, as shown by the decreased number of transformed NIH 3T3 cells caused by an oncogene, takes 19 days and offers the opportunity of studying many potential anticarcinogens.

Protease inhibitors interfere with carcinogenesis by multiple mechanisms, including suppression of oxygen radical formation by neutrophils, suppression of induction of DNA polymerase, and decreasing acids available to the growing cancer cell. Their use as anticarcinogenic agents in man requires investigation.

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