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Hydrogen peroxide degradation and selective carbidopa-induced cytotoxicity against human tumor lines

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Abstract

The carcinoid tumor, an uncommon neuroendocrine neoplasm, is associated with serotonin overproduction as is more common small cell lung carcinoma (SCLC). α-Methyl-dopahydrazine (carbidopa), an inhibitor of the serotonin synthetic enzyme aromatic-L-amino acid decarboxylase, proved lethal to NCI-H727 lung carcinoid cells as well as NCI-H146 and NCI-H209 SCLC cells, but not to five other human tumor cell lines of differing origins [Gilbert JA, Frederick LM, Ames MM. The aromatic-L-amino acid decarboxylase inhibitor carbidopa is selectively cytotoxic to human pulmonary carcinoid and small cell lung carcinoma cells. Clin. Cancer Res. 2000;6:4365–72]. The mechanism of carbidopa cytotoxicity remained an unanswered question. We present data here that incubation of the catechol carbidopa (100 μM) in RPMI and DMEM culture media yielded molar equivalents of hydrogen peroxide (H_2O_2) within 2–4 h. Alkaline elution studies revealed carbidopa-dependent single-strand DNA breaks in sensitive carcinoid cells comparable to those induced by similar concentrations of H_2O_2. Neither compound induced significant DNA damage in carbidopa-resistant NCI-H460 large cell lung carcinoma cells. Furthermore, when carbidopa was incubated with a variety of tumor cell types, not only were decreased media H_2O_2 concentrations detected in the presence of cells, but cell lines least sensitive to carbidopa degraded exogenous H_2O_2 more rapidly than did sensitive cells. Implicated in these studies, pyruvate degraded H_2O_2 in RPMI in a dose- and time-dependent manner and reversed carbidopa-induced cytotoxicity to carcinoid cells. Extracellular pyruvate levels produced per h by resistant large cell lung carcinoma cells averaged four-fold that of sensitive carcinoid cells plated at equal density (24 h time course). Finally, carbidopa exposure (100 μM, 24 h) depleted extracellular pyruvate from sensitive carcinoid cells, but reduced pyruvate levels from resistant NCI-H460 cells less than 17%.

JEL classification: Molecular and Cellular Pharmacology

Keywords: Human; Carcinoid; SCLC; Hydrogen peroxide; Pyruvate

1. Introduction

The carcinoid tumor is a neoplasm of the enterochromaffin system, arising from Kultschitsky cells normally found throughout the gastrointestinal tract, biliary system, pancreas, and bronchial epithelium [1]. The most malignant carcinoids usually originate in the small bowel and metastasize to the liver, often producing symptoms of the carcinoid syndrome [2]. Excessive serotonin produced by the carcinoid tumor is one of the causative agents for the symptoms of this syndrome, which include flushing, diarrhea, and with time, carcinoid heart disease. This laboratory characterized the role of tryptophan hydroxylase (tryptophan 5-monooxygenase; EC 1.14.16.4; TPH) and aromatic-L-amino acid decarboxylase (EC 4.1.1.28; AAAD) in the serotonin biosynthetic pathway of carcinoid tumors [3]. In studies assessing the effect of AAAD inhibitors on human tumor cell lines with a wide range of AAAD activities, we demonstrated that the AAAD inhibitor α-methyl-dopahydrazine (carbidopa) was selectively cytotoxic at μM concentrations to AAAD-rich pulmonary carcinoid and small cell lung carcinoma (SCLC) cells [4]. Upon examination of potential chemotherapeutic applications of these results, sublethal doses of carbidopa produced additive cytotoxic effects in NCI-H727 carcinoid

Abbreviations: TPH, tryptophan hydroxylase; AAAD, aromatic-L-amino acid decarboxylase; carbidopa, α-methyl-dopahydrazine; SCLC, small cell lung carcinoma; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species

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cells in combination with etoposide and cytotoxic synergy in NCI-H146 SCLC cells when coincubated with topotecan [4]. The mechanism of carbidopa cytotoxicity to carcinoid cells, however, was neither clearly apoptotic nor necrotic [4]. Further, as determined utilizing other AAAD and TPH inhibitors, carbidopa cytotoxicity was not directly related to AAAD inhibition or serotonin biosynthesis [4]. The mechanism of carbidopa cytoxicity, therefore, remained unknown.

We report here studies definitively addressing the mechanism of carbidopa cytotoxicity in carcinoid (NCI-H727) cells and resistance in large cell lung carcinoma (NCI-H460) cells. Results are presented implicating formation of toxic breakdown products, specifically the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2), in carbidopa-induced tumor cell death. Data are presented on the role played by cellular degradation of H_2O_2 in the selectivity of carbidopa-induced cytotoxicity to human tumor cell lines. Further, we present evidence suggesting that rapid H_2O_2 degradation by selective cell lines was related to level of tumor cell production over time of the antioxidant pyruvate, an end product synthesized abundantly in the enhanced aerobic glycolysis of highly dedifferentiated cancer cells in the phenomenon known historically as the Warburg effect [5].

2. Materials and methods

2.1. Materials

Carbidopa, 30% H_2O_2, sodium pyruvate, α-methyldopa, α-methyl-*p*-tyrosine, benserazide, catechol, D-dopa, L-dopa, dopamine, norepinephrine, 3-*O*-methyldopa, *p*-chlorophenylalanine, catalase, and superoxide dismutase were purchased from Sigma–Aldrich Chemical Co. All other chemicals used were reagent grade.

2.2. Cell culture

All cell lines were purchased from American Type Culture Collection, and cultured at 37° in a humidified environment of 95%:5% air:CO_2 in the following media (Invitrogen) supplemented with 10% qualified, heat-inactivated fetal bovine serum (Invitrogen): NCI-H146, -H209, -H727, and -H460 lines in RPMI 1640; A204 and MCF7 in DMEM; Du 145 and SK-N-SH in MEM with Earle's salts, 1 mM sodium pyruvate, and 2 mM L-glutamine. L1210 cells were cultured in RPMI with 5% qualified, heat-inactivated fetal bovine serum. Cell culture media free of phenol red, supplemented with 10% qualified, heat-inactivated fetal bovine serum (Invitrogen) and 50 units/ml of penicillin and streptomycin (Invitrogen), was employed for all H_2O_2 and pyruvate studies as follows: RPMI 1640 (Invitrogen 11835), DMEM (Invitrogen 13000 supplemented with 3.7 g/l $NaHCO_3$ as per manufacturer's instructions; however, this item has been discontinued and replaced with 31053, to which 584 mg/l L-glutamine must be added before use as per manufacturer's instructions), and MEM with Earle's salts [Invitrogen 51200 with the non-essential amino acids of the medium packaged separately (Invitrogen 11140) and added just before use] supplemented with 1 mM sodium pyruvate and 2 mM L-glutamine. For media formulations, see http://www.invitrogen.com/content.cfm?pageid=95 and provide the product number. For each individual medium (RPMI, DMEM, MEM), the formulation employed for H_2O_2 and pyruvate studies was identical to that used for cell culture and trypan blue assays with the exception that the former did not contain phenol red.

In experiments to study the effect of individual RPMI media components on the conversion of carbidopa to H_2O_2, RPMI amino acids solution (Sigma R7131), RPMI vitamins solution (Sigma R7256) and individual RPMI inorganic salts and other components (see above website for RPMI formulation) were combined such that the effect of one RPMI ingredient at a time could be tested.

2.3. Trypan blue exclusion assays

Cells (50,000) were seeded in 60 mm plates (Falcon) in 3 ml growth medium supplemented with 10% qualified, heat-inactivated fetal bovine serum (Invitrogen) and 50 units/ml of penicillin and streptomycin (Invitrogen). Following incubation overnight, the test compound or diluent was added to the plate of a suspension cell line or with 3 ml fresh media to a monolayer cell line (20 mM stock carbidopa solution was prepared in DMSO and stored at −20 °C before use). For continuous exposure, drug was replaced daily. Treated cells were collected by centrifugation for 5 min at 4 °C and 200 × *g*, suspension cell lines directly and attached cell lines upon combination of media with monolayer cells lifted with 0.05% trypsin, 0.53 mM EDTA (Invitrogen). After resuspension in culture medium, viable cells were counted on a hemocytometer following a 5 min incubation in 0.2% trypan blue (Invitrogen). IC_{50} values represented the concentration of inhibitor required to bring the number of viable treated cells to 50% of the control cell number upon 72 h of incubation.

Doubling time for cell lines was calculated from experiments ($n \geq 3$) in which untreated cells (50,000) were seeded as described for the trypan blue assay, harvested on multiple successive days, and counted employing the trypan blue method. Doubling time was equal to ln 2/*k*, where *k* is the slope of a linear least-squares regression plot of ln (cell number/original cell number) versus growth time in days.

2.4. H_2O_2 assays

H_2O_2 concentrations in cell culture media were measured with the PeroXOquant Quantitative Peroxide Assay,

aqueous-compatible formulation (Pierce), following centrifugation of media as above to completely remove cells. In experiments measuring the breakdown of H_2O_2 by cultured cells, the cells were plated as for the trypan blue assay, with H_2O_2 added to the media after incubation overnight. The time required to degrade the H_2O_2 concentration by 50% ($t_{1/2}$) was calculated from a linear least-squares regression analysis of a semi-log plot of each time course normalized to data from the time course of media alone.

2.5. Pyruvate assays

Pyruvate levels in cell culture media were measured with the pyruvate kit (Proc. No. 726-UV) from Sigma Diagnostics, Inc., an assay utilizing the enzyme lactate dehydrogenase. The media was first subjected to centrifugation as above to completely remove cells. Initial pyruvate measurements were performed with this method in Mayo Clinic's Department of Laboratory Medicine and Pathology.

2.6. Alkaline elution

Single-strand breaks in DNA of cells exposed to carbidopa or H_2O_2 were measured with the alkaline elution technique of Kohn et al. [6]. Briefly, approximately 5×10^5 [^{14}C]thymidine-labeled (New England Nuclear) experimental cells were placed on a polycarbonate filter (2 μm pore size, Osmonics, Inc.) along with approximately 5×10^5 [^{3}H]thymidine-labeled (New England Nuclear) L1210 cells that had been irradiated (300 rads) for use as an internal standard. The cells were lysed on the filter with 2% SDS and 0.02 M Na_2EDTA, pH 10, and then treated with proteinase K (0.5 mg/ml in the SDS lysis buffer) for 1 min. Elution buffer (tetrapropylammonium hydroxide–0.02 M EDTA, pH 12.1) was drawn through the filter at a rate of 0.057 ml/min and samples were collected in 3 h fractions. The samples were then analyzed for ^{14}C and ^{3}H radioactivity. The apparent DNA single-strand break frequency was determined with calculations previously described [7].

3. Results

3.1. The role of toxic oxidation products in carbidopa-induced cytotoxicity

In previous studies with AAAD-rich carcinoid and SCLC cell lines [4], we noted selective sensitivity to the AAAD inhibitor carbidopa among eight human tumor cell lines. Micromolar concentrations of carbidopa were lethal to three lines, all of lung origin: SCLC NCI-H146, SCLC NCI-H209, and carcinoid NCI-H727 cells, with IC_{50} values (72 h exposure) of 12, 22, and 29 μM, respectively. A moderate decrease in cellular growth (but not complete cell death) was observed with 100 μM carbidopa in the SK-N-SH neuroblastoma and A204 rhabdomyosarcoma tumor cell lines (27 and 42% cell survival, respectively), while no effect was observed in MCF7 breast carcinoma, NCI-H460 large cell lung carcinoma, or Du 145 prostate carcinoma cell lines [4]. Upon determining that carbidopa-induced cytotoxicity was not attributable to inhibition of either AAAD or the serotonin biosynthetic pathway [4], a number of compounds related to carbidopa were examined for their effects on NCI-H727 carcinoid cell growth. As summarized in Table 1, all compounds lethal to carcinoid cells contained an oxidizable catechol moiety within their structure, whereas the compounds having no effect on carcinoid cell growth under identical conditions did not. As catechols are susceptible to autooxidation producing ROS [8], experiments were performed to investigate whether ROS might be involved in the mechanism of carbidopa cytotoxicity. Co-incubation of sensitive NCI-H727 carcinoid cells with the antioxidant enzyme catalase (Fig. 1) prevented cell death induced by carbidopa, consistent with a role for H_2O_2 in carbidopa-induced cytotoxicity.

3.2. H_2O_2 formation and degradation following carbidopa incubation in cell culture media

Formation of H_2O_2 was assessed following carbidopa incubation in the media (RPMI, DMEM, and MEM) used to culture the eight cell lines studied. Incubation of 100 μM carbidopa in RPMI and DMEM resulted in concentrations of approximately 100 μM H_2O_2 after 2–4 h exposure (Fig. 2A). In marked contrast, H_2O_2 concentrations were less than 5 μM after similar incubation periods in MEM (Fig. 2A). When carbidopa incubation in RPMI was repeated with the addition of 1000 U/ml catalase 10 min prior to assaying, no H_2O_2 was detected in the media at any time, confirming identification of the product as H_2O_2 (data not shown). Production of H_2O_2 upon incubation of carbidopa in RPMI medium was accompa-

Table 1
Sensitivity of NCI-H727 carcinoid tumor cell line to test compounds

Test compound	Cell survival after 72 h exposure (%)[a]
α-Methyldopa	0
Benserazide	0
Catechol	0
D-Dopa	0
L-Dopa	0
Dopamine	0
Norepinephrine	0
3-*O*-methyldopa	100
α-Methyl-*p*-tyrosine	100
p-Chlorophenylalanine	100

[a] Concentration = 100 μM; 72 h exposure; 50,000 cells/plate. Cytotoxicity determined by trypan blue exclusion ($n \geq 2$).

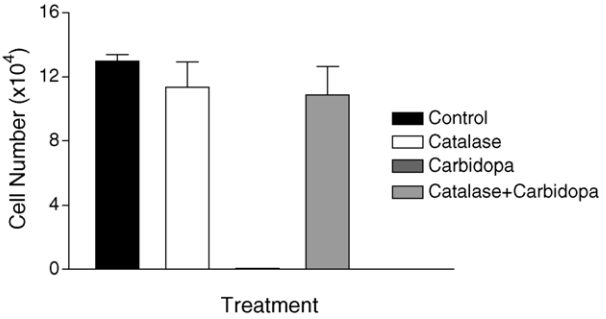

Fig. 1. Effect of catalase on the viability of NCI-H727 carcinoid cells exposed to carbidopa. Cells (50,000/plate) were incubated for 72 h at 37° with 100 μM carbidopa and 1000 U/ml catalase. The number of viable cells in each plate was determined with the trypan blue exclusion assay described in Section 2. These data represent the mean ± S.E.M. of triplicate experiments.

nied by the disappearance of the parent drug as measured by HPLC (data not shown). Additionally, omission of the sodium bicarbonate component of the media buffering system prevented the conversion of carbidopa to H_2O_2 (data not shown).

When 100 μM exogenous H_2O_2 was incubated in each of the three media, H_2O_2 concentrations were reduced 30–40% after 2–4 h in RPMI and DMEM, while no H_2O_2 was detectable at any time following addition to MEM medium (Fig. 2B). Because any resistance to carbidopa of cells cultured in MEM (SK-N-SH and Du 145) may well be due to the rapid degradation of H_2O_2 by the medium rather than to cellular effects, these two cell lines were omitted from further studies.

3.3. Formation of DNA single-strand breaks upon incubation of carbidopa or H_2O_2 with human tumor cell lines

Exposure of tumor cells to H_2O_2 results in DNA single-strand breaks measurable with the alkaline elution assay [9–11]. Given the presence of H_2O_2 in cell culture media after carbidopa incubation, we investigated whether H_2O_2 might play a central role in carbidopa cytotoxicity to sensitive cells as measured by DNA damage. We evaluated formation of DNA single-strand breaks in a sensitive cell line (carcinoid NCI-H727) and in a resistant cell line (large cell lung carcinoma NCI-H460) following exposure to carbidopa and to H_2O_2. Substantial DNA damage was observed upon 7 h incubation of carbidopa at concentrations greater than the IC_{50} with the sensitive carcinoid cells, but not with the resistant large cell lung carcinoma cells (Fig. 3A). The 7 h exposure was selected to assure DNA damage was not due to cell death, observed no sooner than 12 h [4]. Similar DNA damage dose–response curves were obtained upon incubation of both cell lines with equimolar concentrations of H_2O_2 (Fig. 3B). Of note, under similar conditions, exposure of cells to 100 μM carbidopa (7 h) resulted in the production of 60 μM

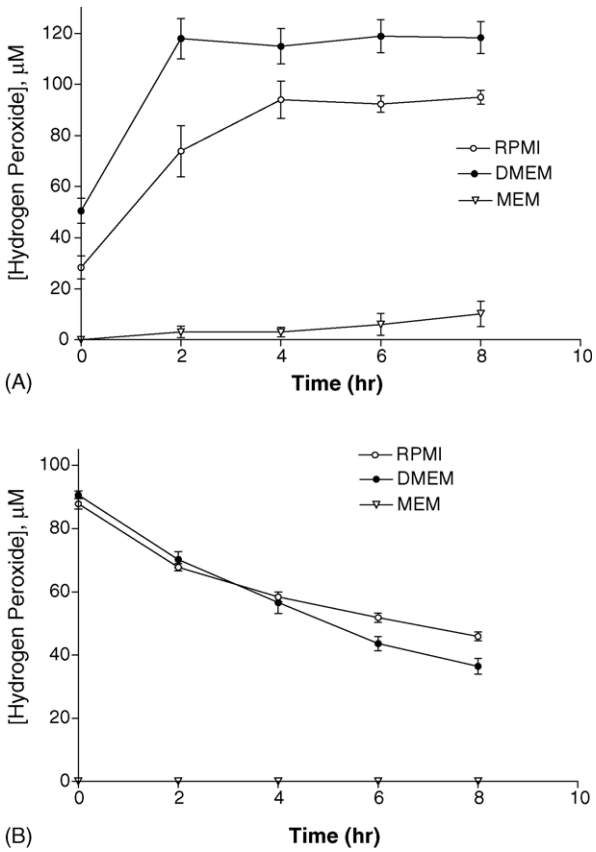

Fig. 2. (A) Time course of H_2O_2 formation upon carbidopa incubation in culture media. Carbidopa (100 μM) was added to media without phenol red, incubated for 8 h at 37°, and the concentration of H_2O_2 in the media was measured at the indicated times as described in Section 2. (B) Time course of H_2O_2 degradation in culture media. H_2O_2 (100 μM) was added to media without phenol red, incubated for 8 h at 37°, and its concentration then measured at the indicated times as described in Section 2. These data represent the mean ± S.E.M. of triplicate experiments.

H_2O_2 in the media of carcinoid cells while less than 5 μM H_2O_2 was detected in the media of resistant large cell lung carcinoma cells (data not shown). The modestly lower level of damage observed with H_2O_2 treatment of carcinoid cells compared to carbidopa exposure most likely reflected repair of DNA lesions and/or gradual disappearance of H_2O_2 following exposure of cells to a single dose of H_2O_2, while carbidopa treatment released H_2O_2 over time thereby providing continuing exposure (see Fig. 2A and B). Nonetheless, the similarity of carcinoid DNA damage levels induced by equimolar concentrations of carbidopa and H_2O_2, along with the lack of damage from either agent to NCI-H460 DNA, are consistent with a critical role for H_2O_2 in carbidopa cytotoxicity.

3.4. H_2O_2 degradation following incubation with tumor cells

As the patterns of H_2O_2 formation and degradation in RPMI and DMEM were similar (see Fig. 2A and B), differences in sensitivity to carbidopa/H_2O_2 cytotoxicity in human tumor cell lines cultured in these media should

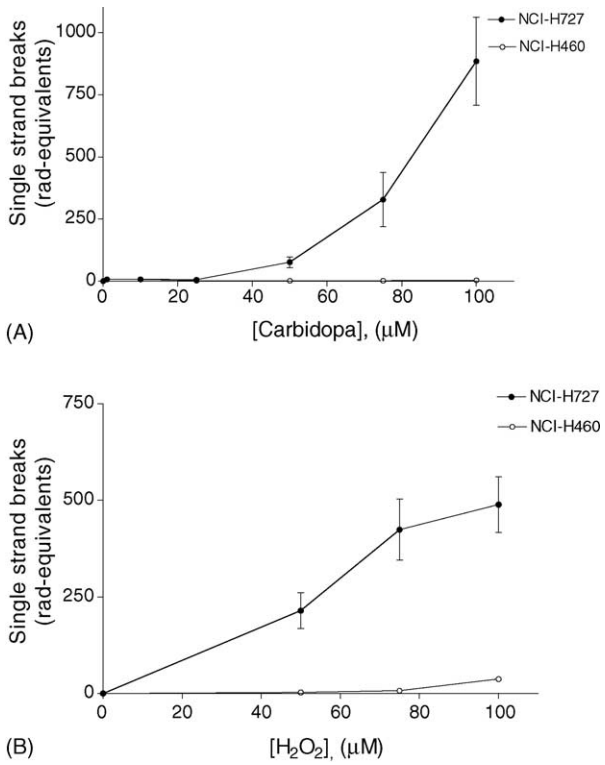

Fig. 3. (A) Effect of increasing carbidopa concentrations on single-strand DNA breaks in NCI-H727 carcinoid and NCI-H460 large cell lung carcinoma cells. Carcinoid or large cell lung carcinoma cells were incubated with increasing concentrations of carbidopa in RPMI for 7 h at 37°, and single-strand DNA breaks were measured in duplicate samples with the alkaline elution assay as described in Section 2. (B) Effect of increasing H_2O_2 concentrations on single-strand DNA breaks in NCI-H727 carcinoid and HCI-H460 large cell lung carcinoma cells. Carcinoid or large cell lung carcinoma cells were incubated with increasing concentrations of H_2O_2 in RPMI for 7 h at 37°, and single-strand DNA breaks were measured with the alkaline elution assay. These data represent the mean ± S.E.M. of triplicate experiments.

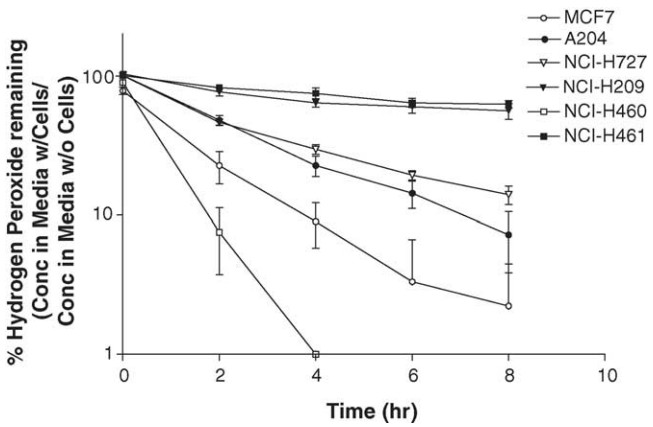

Fig. 4. Time course of H_2O_2 breakdown in culture media with cells. Cells (50,000/plate) were plated and incubated overnight as for the trypan blue assay, except that the media did not contain phenol red. H_2O_2 (100 μM) was added to the media, incubated at 37° for 8 h, and its concentration in medium then measured at the indicated times as described in Section 2. These data represent the mean ± S.E.M. of triplicate experiments, with each time course normalized to data from the time course of media alone.

reflect differences in cellular properties. In initial time course studies of carbidopa incubation with the six tumor cell lines of interest, H_2O_2 levels detected in media alone were always higher than that in media containing cells, suggesting cellular degradation of H_2O_2 (data not shown). We therefore assessed the ability of the six cell lines to degrade H_2O_2 with correction for degradation by the media alone. Upon incubation with cells, 100 μM H_2O_2 was degraded more than 90% in 2 h by NCI-H460 cells and in 4 and 8 h by MCF7 and A204 lines, respectively, but decreasing levels of degradation were observed during that period for NCI-H727, NCI-H209, and NCI-H146 cells (Fig. 4). Comparison of these data with the carbidopa cytotoxicity data demonstrated that the cell lines least sensitive to the drug degraded H_2O_2 much more rapidly than did the most sensitive cells.

3.5. Pyruvate degradation of H_2O_2

The rapid degradation of H_2O_2 seen in MEM instigated a search for the causative agent by comparing the composition of MEM versus RPMI and DMEM. The most potentially relevant difference between MEM and the latter two media was the presence of 1 mM pyruvate in MEM but the absence of this compound in RPMI and DMEM [11]. Verifying the documented antioxidant activity of pyruvate [11], addition of pyruvate to RPMI containing 100 μM H_2O_2 reduced the half-life of H_2O_2 in a concentration-dependent manner (Table 2). Specifically, pyruvate concentrations $\geq$50 μM produced rapid H_2O_2 breakdown, i.e., a half-life less than 4 h. A pyruvate concentration of 200 μM was required to degrade 100 μM H_2O_2 to <1% in 2 h. Accordingly, when sensitive NCI-H727 carcinoid cells were incubated (24 h) with 60 μM carbidopa (~two times IC_{50}) and 250 μM pyruvate, carbidopa cytotoxicity was abolished ($11.23 \pm 1.85 \times 10^4$ treated cells versus $12.30 \pm 0.52 \times 10^4$ control cells; $n = 3$, mean ± S.E.M.).

We next measured pyruvate concentrations in RPMI and DMEM media (which do not contain pyruvate) incubated with the six untreated tumor cell lines (Table 3). After 24 h, concentrations of extracellular pyruvate effective in rapid H_2O_2 breakdown (~50 μM) were only noted for the MCF7 and NCI-H460 cells, both lines resistant to carbidopa. Also

Table 2
H_2O_2 degradation by pyruvate

Pyruvate concentration (μM)	H_2O_2 half-life (h)[a]
25	>8
50	3.40 ± 0.04
100	1.15 ± 0.02
150	0.61 ± 0.05
200	0.37 ± 0.02

[a] H_2O_2 concentrations (initially 100 μM in RPMI) measured as described in Section 2. Half-life calculated by linear least-squares regression analysis of semi-log plot of each time course normalized to data from time course of RPMI alone (mean ± S.E.M.; $n = 3$).

Table 3
Human tumor cell lines: production of pyruvate and doubling time

Cell line	[Pyruvate] (μM)[a]	Doubling time (days)
NCI-H146	29.0 ± 1.0	2.58
NCI-H209	32.7 ± 3.3	2.60
NCI-H727	33.5 ± 3.8	2.81
A204	21.0 ± 1.5	1.40
MCF7	49.5 ± 7.5	1.26
NCI-H460	48.0 ± 2.5	0.64

[a] Cells (50,000/plate) were incubated at 37 °C for 24 h following seeding. The media from the plate was then assayed for pyruvate as described in Section 2 (mean ± S.E.M.; $n = 3$). Cell lines were cultured in the following media supplemented with 10% fetal bovine serum: NCI-H146, -H209, -H727, and -H460 lines in RPMI 1640, and A204 and MCF7 in DMEM.

included in Table 3 are the doubling times determined for the six cell lines. The cell lines least sensitive to carbidopa, which were also the most effective in rapidly degrading H_2O_2 (see Fig. 4), proved also to have the shortest doubling times. These data suggested that doubling time, coupled with extracellular pyruvate measurement, might be important predictors of the ability of the six cancer lines to degrade the H_2O_2 accumulated during the 72 h carbidopa exposure employed in cytotoxicity assays. When pyruvate production by tumor cells was compared for one untreated sensitive (carcinoid) and resistant (large cell lung carcinoma) cell line over a 24 h period, extracellular pyruvate levels produced per h by the resistant tumor cells averaged four-fold that of the sensitive cells (Fig. 5). During the course of this experiment, the mean number of carcinoid cells increased to 62,000/plate from an original 50,000/plate while the large cell lung carcinoma number rose to 438,000/plate, suggesting a relationship between extracellular pyruvate production and growth of the tumor cell line. Furthermore, after exposure (24 h) of sensitive carcinoid cells and resistant NCI-H460 cells to 100 μM carbidopa, extracellular pyruvate was undetectable in media of sensitive cells but levels from resistant cells were only reduced <17% (Fig. 6). Thus, the amount of pyruvate produced over time may be important in carbidopa-resistant tumor cells to counteract the levels of H_2O_2 formed during the same period from carbidopa autooxidation.

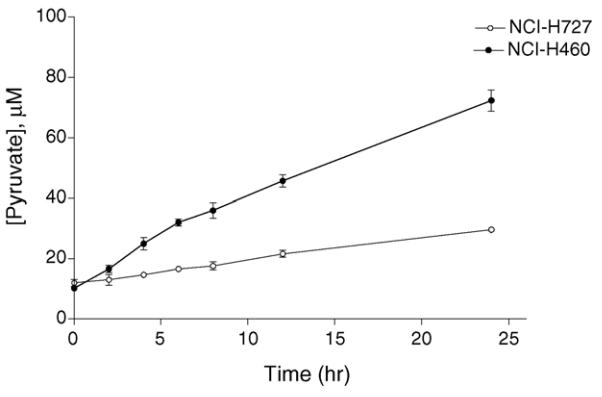

Fig. 5. Time course of pyruvate formation in culture media from untreated NCI-H727 carcinoid and HCI-H460 large cell lung carcinoma cells. Cells (50,000/plate) were plated and incubated overnight as for the trypan blue assay, except that the RPMI did not contain phenol red. The pyruvate levels in the media were then determined at the indicated times as described in Section 2. These data represent the mean ± S.E.M. of triplicate experiments.

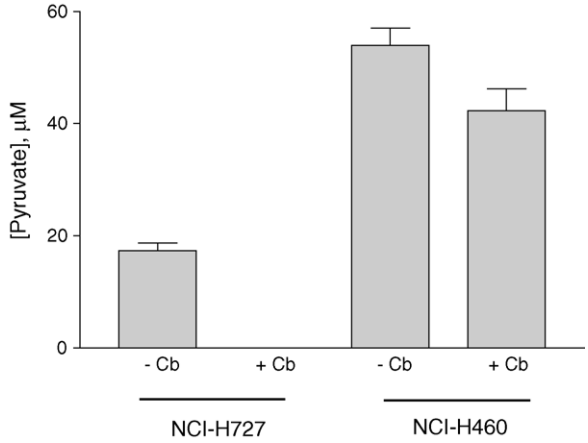

Fig. 6. Effect of 24 h incubation with carbidopa on pyruvate levels in culture media from NCI-H727 carcinoid and NCI-H460 large cell lung carcinoma cells. Cells (50,000/plate) were plated, allowed to attach overnight, and incubated for 24 h at 37° with/without 100 μM carbidopa as for the trypan blue assay, except that the RPMI did not contain phenol red. The pyruvate levels in the media were then determined as described in Section 2. These data represent the mean ± S.E.M. of triplicate experiments.

4. Discussion

While assessing the feasibility of targeting highly expressed AAAD in carcinoid and lung cancers with selective antitumor agents, the AAAD inhibitor carbidopa was found to be uniquely lethal to three serotonergic lung lines (NCI-H727 carcinoid and NCI-H146 and NCI-H209 SCLC cells) of the eight human tumor cell lines evaluated in that study [4]. The mechanism of carbidopa cytotoxicity, however, remained unknown, as we determined that carbidopa did not induce cytotoxicity by inhibiting AAAD or interrupting serotonin synthesis [4]. Data shown in this report document the oxidation of the catechol-containing carbidopa to the ROS H_2O_2. A central role for H_2O_2 in carbidopa cytotoxicity was supported by catalase rescue of carbidopa-treated cells, by the similarity in levels of DNA damage induced by equimolar concentrations of carbidopa and H_2O_2, and by findings that carbidopa was most toxic to cell lines least able to quickly degrade H_2O_2. Our studies suggested that H_2O_2 concentrations to which cells are exposed at any given time result from a balance between production of H_2O_2 from carbidopa and, importantly, degradation of H_2O_2 by tumor cells. Our data also suggested that the antioxidant pyruvate plays a role in protecting tumor cells from carbidopa (and thus H_2O_2) cytotoxicity. In particular, over a 24 h period, sensitive (carcinoid) cells produced one-fourth the hourly pyruvate levels of resistant (large cell lung carcinoma) cells (a factor

possibly related to growth rate) and, in contrast to resistant cells, were unable to maintain extracellular pyruvate levels during 24 h exposure to the autooxidizing carbidopa.

Catechols are susceptible to autooxidation (review [8]), and the toxic effects of the catechol dopa on cultured neuronal and tumor cells have been reported for almost 20 years to result from its autooxidation to the ROS superoxide radical and H_2O_2 [12–17]. This, however, is the first report on the autooxidation of the AAAD inhibitor carbidopa and on the role played by pyruvate, an end product of enhanced aerobic glycolysis in highly dedifferentiated cancer cells (a phenomenon known as the Warburg effect), in providing antioxidant protection to selective tumor lines.

Pyruvate, like other α-keto-carboxylic acids, reacts non-enzymatically with H_2O_2 in aqueous solutions [18], and numerous reports describe the in vitro protective effects of pyruvate on cells sensitive to the oxidative effects of H_2O_2 [11,19–24]. Pyruvate is regulated and utilized differently in cancer cells than in normal cells. It has long been recognized that, compared to normal cells, cancer cells display high levels of glycolytic metabolism even in the presence of oxygen (known as the Warburg effect) (see [5]). Cancer cells display varying degrees of differentiation from highly differentiated, demonstrating similarity to cells of the organ of origin with relatively normal glycolysis and slow growth, to highly dedifferentiated, exhibiting enhanced glycolysis and rapid growth (see review [25]). Baggetto [25] summarized evidence that the regulation of glycolytic and oxidative pathways differs in normal and malignant cells. In particular, fast-growing, poorly differentiated cancer cells characteristically show elevated levels of aerobic glycolysis. Furthermore, highly glycolytic cancer cells display aberrations in the normal metabolic pathways. Important to our studies is the observation that because glutamine, rather than glucose, is a major oxidizable substrate in cancer cells, more than 90% of pyruvate produced during glycolysis is not subsequently employed by mitochondria for energy production via the Krebs cycle and therefore accumulates in the cytosol or is converted to lactate.

This enhanced production of pyruvate by very glycolytic, highly dedifferentiated cancer cells may provide a survival advantage by affording antioxidant protection against environmental oxidative insult. Furthermore, pyruvate has been shown to activate hypoxia-inducible gene expression (linked to malignant transformation) independently of hypoxia [26] as well as induce angiogenesis in in vitro and in vivo assays [27]. All of these described activities, taken together, suggest that pyruvate, the end product of glycolysis, might be a significant contributor to tumor growth. The studies described here also suggest that this link between increased tumor cell proliferation and enhanced aerobic glycolysis might play a role in the selectivity of carbidopa-induced cytotoxicity towards cell lines which are unable to quickly degrade hydrogen peroxide. While our data suggest the importance of extracellular pyruvate in the protection of resistant human cancer cell lines from H_2O_2 generated by carbidopa, they do not exclude the possible involvement of additional antioxidant mechanisms within intact tumor cells. Any analyses of the antioxidant arsenal available to carbidopa-resistant tumor lines should begin, however, with consideration of intracellular pyruvate, e.g., determining the time required to reach equilibrium between extracellular and intracellular pyruvate concentrations and whether equilibration occurs with cells growing logarithmically; ascertaining whether pyruvate efflux in cancer cells is diffusion- or transporter-driven and if limitations are involved; assessing whether cytosolic pyruvate pools exist in these cancer lines as reported for other cell types (including glioma [28]) and if compartmentation affects the equilibration of intracellular and extracellular pyruvate levels. All of these variables could potentially act to enhance the effectiveness of intracellular pyruvate in degrading H_2O_2 in comparison to extracellular pyruvate (free in solution).

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