INHIBITION OF TUMORAL CELL RESPIRATION AND GROWTH BY NORDIHYDROGUAIARETIC ACID

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Abstract—The effects of nordihydroguaiaretic acid (NDGA), best known as an inhibitor of lipoxygenase activities, on the culture growth, oxygen consumption, ATP level, viability, and redox state of some electron carriers of intact TA3 and 786A ascites tumor cells have been studied. NDGA inhibited the respiration rate of these two tumor cell lines by preventing electron flow through the respiratory chain. Consequently, ATP levels, cell viability and culture growth rates were decreased. NDGA did not noticeably inhibit electron flow through both cytochrome oxidase and ubiquinone-cytochrome b–c_1 complex. Also, the presence of NDGA changed to redox state of $NAD(P)^+$ to a more reduced level, and the redox states of ubiquinone, cytochrome *b* and cytochromes $c + c_1$ changed to a more oxidized level. These observations suggest that the electron transport in the tumor mitochondria was inhibited by NDGA at the NADH-dehydrogenase-ubiquinone level (energy-conserving site 1). As a consequence, mitochondrial ATP synthesis would be interrupted. This event could be related to the cytotoxic effect of NDGA.

Key words: cellular growth; tumor cell respiration; ATP level; inhibition; nordihydroguaiaretic acid

The polyhydroxyphenolic antioxidant NDGA‖ was once generally used at levels of 0.01–0.02% to prevent changes in flavor and nutritive values which result from oxidation of unsaturated fats and fat-containing products [1, 2]. A remarkably low LD_{50} of 4000 and 5500 mg/kg (orally) has been reported in mice and rats, respectively [2]; indeed, an LD_{50} value of 800 mg/kg has been found in mice upon the intraperitoneal injection of NDGA, using peanut oil as the vehicle [1]. Prolonged feeding of 0.5–1.0% NDGA in the total diet for 74 weeks had a toxic effect on the reticuloendothelial system and the kidney tubules of the rat [2]. Consequently, there are few countries today where its use as a food additive is still permitted [2]. However, NDGA induced no gross or organ-specific toxicity in rats fed a semi-purified diet for 200 days [3]. Moreover, injection of 950 mg NDGA/kg into pregnant rabbits resulted in reduced hydroxyurea developmental toxicity in fetuses, which was manifested by greatly reduced incidences of specific malformations and diminished severity of some hydroxyurea-induced defects [4].

NDGA is perhaps best known for its inhibitory activity at low concentrations, on the lipoxygenase pathways of arachidonic acid metabolism and, hence, leukotriene synthesis. Also, at higher concentrations, it inhibits cyclooxygenase and, therefore, prostanoid production [5]. Consequently, the proliferation of normal and malignant cells observed in culture is suppressed by NDGA with no effect on cell viability, either when these cells are exposed to various growth factors, hormones, and fatty acids and their derivatives [6–10], or when certain eicosanoids are synthesized by the cells themselves [6, 10–13]. Moreover, this compound is also able to inhibit the growth of malignant cells, since it inhibits ornithine decarboxylase activity induced by mitogens or tumor promoters [14–16].

In addition, NDGA is antimutagenic and anti-tumorigenic, which may be due to its multiple effects as an inhibitor of carcinogen metabolism and DNA–adduct formation, and as a free radical scavenger and, thus, it is capable of inhibiting enzymatic and non-enzymatic oxidative reactions [3, 15–23].

On the other hand, it has been reported that NDGA inhibits electron flow and energy transfer in isolated mammalian mitochondria [24–26]. The probable sites of inhibition of mitochondrial electron transport are the NADH-coenzyme Q reductase and succinate-coenzyme Q reductase systems [24]. It is also an inhibitor of glucose absorption in rat intestine [5], glycolysis [1,27] and respiration of a wide variety of malignant cells and other cancer tissues, both *in vitro* and *in vivo* [1,28], as well as the respiration of the TA3 and the 786A ascites cell lines in the presence and absence of the uncoupler CCCP [29].

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‖ Abbreviations: NDGA, nordihydroguaiaretic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; and TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylene diamine.

These effects on the growth and respiration of several tumor cell lines suggest that NDGA may have antineoplastic action, which would be mediated through a process that is not connected with eicosanoid production or with ornithine decarboxylase activity. It is more likely that it might interfere with the oxidative process and energy metabolism of the tumor cells. We present evidence to suggest that NDGA inhibits the mitochondrial electron flow in intact cells between NADH-dehydrogenase and ubiquinone, which results in a pronounced reduction of ATP levels and, consequently, the inhibition of the growth ascites tumor cells.

MATERIALS AND METHODS

Chemicals. Tris–HCl, antimycin, CCCP, a diagnostic kit for the determination of ATP levels, EGTA, fetal bovine serum, glutamine, duroquinone, HEPES, NDGA, rotenone and TMPD were purchased from the Sigma Chemical Co. (St. Louis, MO). The stock solution of NDGA was prepared in ethanol or DMSO; no effects of these solvents at the concentrations used in our experiments were observed. Duroquinol was prepared from duroquinone in alcoholic solution by reduction with sodium borohydride; it was recrystallized, and the stock solution was dissolved in DMSO, as described by Boveris *et al* [30]. All other reagents were of the highest purity commercially available.

Harvesting of tumor cells. The following ascites tumors were grown by weekly intraperitoneal injection into the appropriate tumor bearing-mice: the 786A ascites tumor (sarcoma) was carried in young adult male A Swiss mice and the TA3 ascites tumor (carcinoma) was propagated in young adult male CAF 1 Jax mice. All animals were fed with a standard laboratory chow and water *ad lib*. The tumor cells were harvested 7–9 days after intraperitoneal inoculation of ascites fluid from donor mice by centrifugation of 100 *g* for 5 min at 4°. Then the cells were washed twice with 150 mM NaCl, 5 mM KCl and 10 mM Tris–HCl, pH 7.4, essentially as described by Moreadith and Fiskum [31]. The tumor cells were resuspended in the same medium at a concentration of 30–35 mg protein/mL. The cells appeared to be virtually free of erythrocytes and other contaminants, and showed a viability of 95–98%, as indicated by exclusion of trypan blue and determined as follows: a sample of the cell suspension was mixed with 0.5% trypan blue (final concentration) in isotonic saline solution, and the number of viable and nonviable cells was counted in an Improved Neubauer counter chamber. About 150–200 cells were examined. The protein concentration was determined by a modified biuret reaction standardized with serum albumin [32].

Inhibition of the growth of 786A and TA3 cell lines. Both cell lines were cultured in the absence or presence of NDGA in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 25 mM HEPES, 44 mM $NaHCO_3$, penicillin (100 U/mL) and streptomycin (100 μg/mL). For the experiments, 1.8 to 2.2×10^5 cells/mL were seeded in 30 mL of culture medium, using 100-mL culture flasks and grown at 37° for up to 96 hr. The cells were allowed to grow for 24 hr, and then NDGA was added [29]. Cell numbers were determined with a Neubauer counting chamber every 24 hr, as described above.

Cell respiration. Oxygen uptake was measured polarographically at 25° with a Clark electrode No. 5331 (Yellow Springs Instrument) and using a YSI model 53 monitor linked to a 100 mV monochannel Goerz RE 511 recorder. The 2.0-mL reaction mixture contained 150 mM NaCl, 5 mM KCl and 10 mM Tris–HCl, pH 7.4, plus 5 mM glutamine as substrate and 2.5 mg protein/mL of ascites tumor cells [29].

Spectrophotometric determinations. The redox state of the respiratory carriers was monitored at 25° by dual-wavelength spectrophotometry (Aminco DW-2). The 2.5-mL reaction medium consisted of 150 mM NaCl, 5 mM KCl, 10 mM Tris–HCl (pH 7.4) and 5 mM glutamine as substrate. The wavelength couples used were: 340–390 nm for NAD(P)H [33], 275–245 nm for ubiquinone [34], 430–410 nm for cytochrome *b* [35] and 550–540 nm for cytochromes $c + c_1$ [34]. The cuvette, with a 1.0-cm light path, was equipped with magnetic stirring. When added, NDGA (dissolved in ethanol), as indicated in the figures, was injected rapidly from microsyringes in such a way as to achieve the shortest possible mixing time [29].

Cellular ATP levels. Tumor cells (10^7/mL) were shaken at 37° (75 oscillations/min) in phosphate-buffered saline (pH 7.4), containing 5% fetal bovine serum, 2 mM EGTA and supplemented with 5 mM glutamine and 10 mM glucose as substrates in order to maintain a constant steady-state level of cellular ATP for the duration of the incubations [36], either with or without NDGA. Aliquots of cell suspension (1.0 mL) were removed at various times for processing and spectrophotometric assay of cellular ATP content, using a diagnostic kit as recommended by the manufacturer without modifications. At the same time, 0.2- and 2.0-mL aliquots of cell suspension were removed to determine both viability and cellular respiration, respectively, as described above.

RESULTS

We have reported previously that NDGA is an inhibitor of the mitochondrial electron flow of tumor cells [29]. To establish more precisely the mechanism of action of NDGA and its consequences, we examined the effect of this antioxidant on the growth, ATP content, viability, respiration and redox states of some electron carriers in tumor cell lines. Figure 1 shows the effect in culture of different concentrations of NDGA on the percentage survival of TA3 and 786A ascites tumor cell lines exposed for periods up to 72 hr. For each concentration of NDGA tested, toxicity increased with the time of exposure to the chemical. At higher NDGA concentrations, the inhibition of the growth of both ascites tumor cell lines was stronger. No noticeable variation between the two cell lines used was found in the levels of survival of cells for each NDGA concentration tested.

Addition of NDGA to the incubation medium of ascites tumor cell lines resulted in a number of

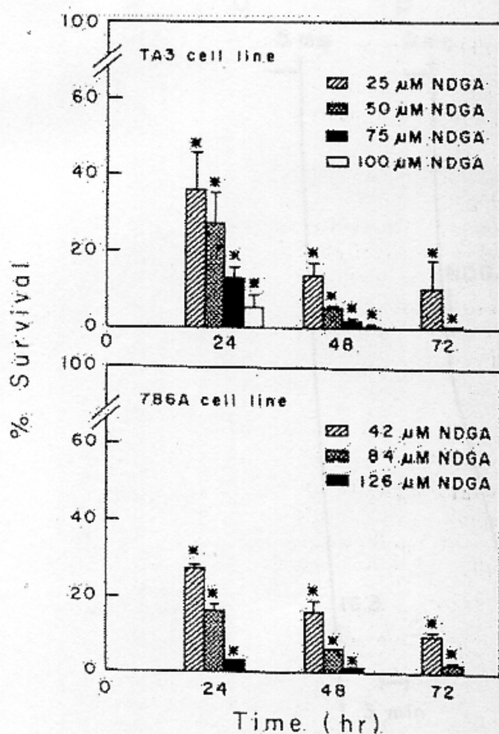

Fig. 1. Percentage of survival of TA3 and 786A cell lines cultured for periods up to 72 hr in the presence of NDGA. Each result is the mean ± SD of four experiments, with each assay performed in quadruplicate. Significance was determined by comparing the respective control value with that of the NDGA-treated cells (Student's *t*-test; * = P < 0.001).

related metabolic effects, which increased with the time of exposure to this chemical in a concentration-dependent manner. Within 1 hr after the addition of 250 μM NDGA (Fig. 2), cellular respiration and the ATP level of TA3 ascites tumor cells were already strongly decreased to 43 and 42% of respective control values (Fig. 2A and B). However, viability diminished less rapidly; a decrease to 78% of the control value was observed after 1 hr (Fig. 2C). Probably the complex chain of events that result in cell death would require a certain period of time. After 4 hr of incubation with NDGA, oxygen consumption, ATP level and viability were decreased to 25, 18 and 26% of respective control values (Fig. 2). These results indicate that NDGA was able to affect mitochondrial oxidative phosphorylation in intact cells, which could explain, in part, the cytotoxic effect of NDGA. No noticeable variation was found in respiration rate, ATP levels and viability between the two cell lines used (results not shown).

A representative polarographic trace of the respiratory rate of the TA3 cell line is shown in Fig. 3a; the addition of NDGA produced an inhibition of the rate of oxygen consumption (Fig. 3b). The effects of NDGA concentration on the respiratory rates of 786A and TA3 cell lines are reported in Fig. 4. Very similar sigmoidal inhibitory curves were observed when the NDGA concentration was increased in the assay system with these two tumor cell lines, both in the absence and in the presence of the uncoupler CCCP. Maximal inhibition (93–96%) of the respiratory rates of both tumor cell lines in the absence of CCCP was attained at about 0.44–0.59 mM. The I_{50} values were 0.21 and 0.24 mM NDGA for the TA3 and 786A cell lines, respectively. Maximal inhibition (98–100%) of CCCP-stimulated respiration by 786A and TA3 cell lines was obtained at about 0.44 to 0.50 mM NDGA. The I_{50} values were about 0.20 and 0.22 mM for the TA3 and 786A

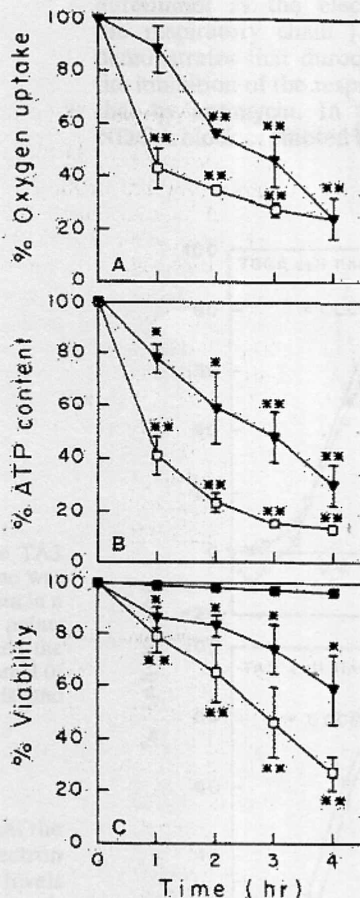

Fig. 2. Time course of the effect of NDGA on the decrease of (A) oxygen consumption rate, (B) ATP content and (C) viability of the TA3 ascites cell line. Cells were incubated at 37° in the absence or presence of (▼) 150 μM and (□) 250 μM NDGA. Determinations were made after incubation for the lengths of time indicated. Results are expressed as the mean percentage of the respective control ± SD of four to six independent experiments. At zero time, the mean oxygen consumption rate ± SD of control cells was 4.35 ± 0.17 nmol 0/min10^6 cells; for ATP content, the control value was 9.39 ± 1.76 nmol ATP/10^6 cells. Viability is expressed as the percentage of trypan blue-excluded cells vs total amount of cells present in 1 mL. (■) Viability in the absence of NDGA. At zero time, the mean cell viability ± SD was $10 \times 10^6 \pm 1.7 \times 10^5$. Incubation had little effect on these parameters. Significance was determined by comparing the respective control value with that of the NDGA-treated cells (Student's *t*-test; * = P < 0.007; ** = P < 0.001).

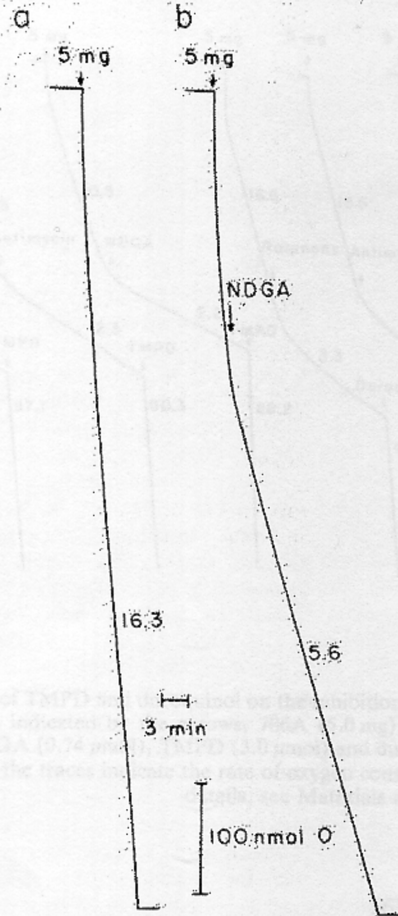

Fig. 3. Effect of NDGA on the respiration of the TA3 ascites tumor cell line. In *a* and *b*, 5 mM glutamine was used as substrate. Addition of cells (5.0 mg of protein in *a* and *b*) and NDGA (0.24 mM in *b*) was made at the points indicated by the arrows. The numbers placed beside the curves indicate the rate of oxygen consumption in nmol 0/min/mg protein. For other details, see Materials and Methods.

cell lines, respectively. These results suggest that the NDGA specifically inhibits mitochondrial electron flow. No noticeable variation was found in the levels of inhibition between the two cell lines used, although at NDGA concentrations below 0.1 mM a very slight uncoupler effect could be observed on the 786A cell line tumor (10% stimulation). A similar effect of NDGA on NADH oxidase activity of heavy beef heart mitochondria was found by Pardini *et al.* [24]. Many phenols have been shown to uncouple oxidative phosphorylation [37], but NDGA is a better electron flow inhibitor than uncoupler.

To establish more precisely the inhibitory sites of NDGA within the mitochondrial electron transport chain of intact tumor cells, we studied the effect of NDGA on oxygen consumption when well-characterized synthetic substrates that donate electrons to the energy-conserving sites 2 and 3 were added. The effect of NDGA on the electron flow through cytochrome oxidase was examined by appraising the ability of TMPD to bypass the respiratory inhibition originated by NDGA. This approach is based on the principle that Wurster's blue (WB^+), which is the oxidized form of TMPD, acts as an electron acceptor from ubisemiquinone; then the TMPD thus formed donates electrons to cytochrome *c*, permitting the cytochrome oxidase reaction [38]. The traces presented in Fig. 5 show that the inhibition of oxygen consumption by rotenone, antimycin and NDGA was bypassed by TMPD. These results demonstrate that the site of inhibition of electron flow by NDGA is not at the cytochrome oxidase step. The action of NDGA on the cytochrome b–c_1 complex was studied by using duroquinol as the electron donor to site 2 of the respiratory chain [30, 39, 40]. Figure 5 also demonstrates that duroquinol completely reversed the inhibition of the respiration by rotenone but not that by antimycin. In return, the bypass of the NDGA block promoted by duroquinol attained only

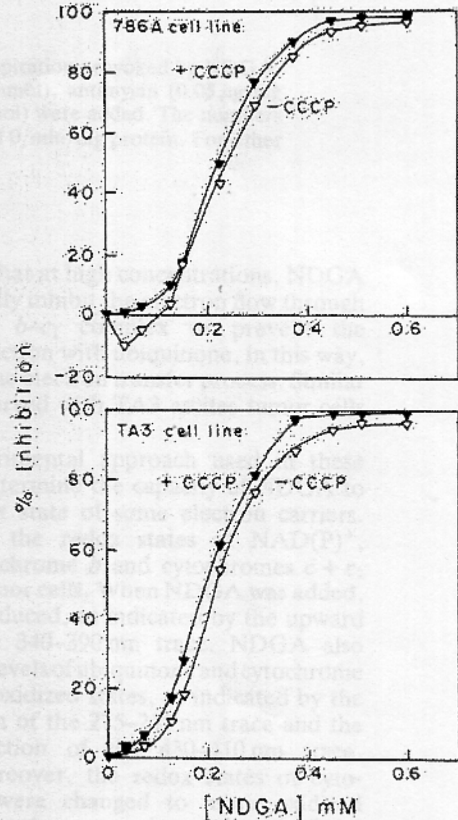

Fig. 4. Effect of NDGA concentration on the respiration of 786A and TA3 cell lines in the presence and absence of the uncoupler CCCP. Results are expressed as percent inhibition of oxygen consumption in the absence of inhibitor; control activities were 15.6 (0.07 μM CCCP) and 12.3 (−CCCP), 20.2 (0.07 μM CCCP) and 16.5 (−CCCP) nmol 0/min/mg protein in 786A and TA3 cell lines, respectively. The inhibitory effects of different NDGA concentrations were obtained from experiments like those of Fig. 3. Each point is the mean of four to six independent experiments. Other conditions were as in Fig. 3.

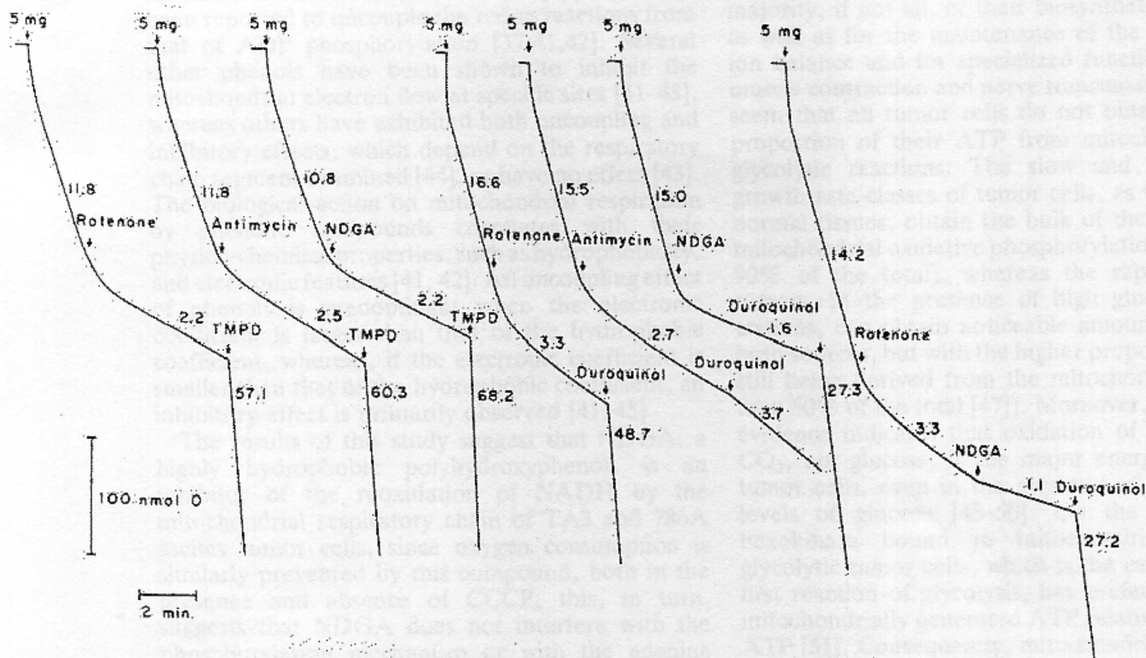

Fig. 5. Effect of TMPD and duroquinol on the inhibition of 786A cell respiration provoked by NDGA. At the points indicated by the arrows, 786A (5.0 mg), rotenone (0.4 nmol), antimycin (0.05 μg/mg protein), NDGA (0.74 μmol), TMPD (3.0 μmol) and duroquinol (2.8 μmol) were added. The numbers placed beside the traces indicate the rate of oxygen consumption in nmol 0/min/mg protein. For other details, see Materials and Methods.

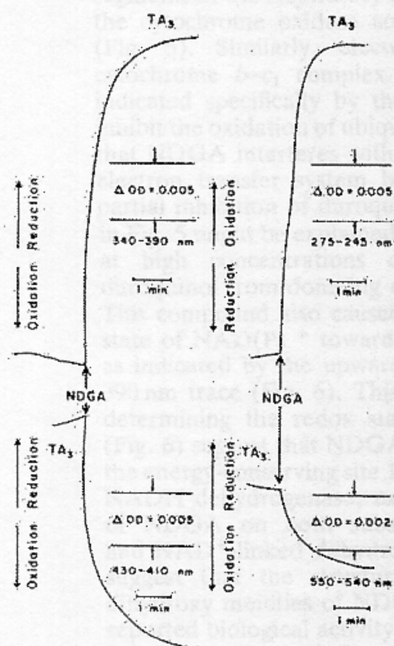

Fig. 6. Effect of NDGA on the redox states of NAD(P)$^+$, ubiquinone, cytochrome b and cytochromes $c + c_1$ in TA3 cell lines. At the points indicated by the arrows, 2.0 μmol NDGA was added to the reaction medium. Tumor cells used were: 12.5 mg for NAD(P)$^+$, 5.0 mg for ubiquinone, and 13.0 mg of protein for cytochromes. For other experimental conditions, see Materials and Methods.

56%, indicating that at high concentrations, NDGA may either partially inhibit the electron flow through the cytochrome b–c_1 complex or prevent the duroquinol interaction with ubiquinone, in this way, interfering with the electron transfer process. Similar results were observed with TA3 ascites tumor cells (data not shown).

Another experimental approach used in these studies was to determine the capacity of NDGA to change the redox state of some electron carriers. Figure 6 shows the redox states of NAD(P)$^+$, ubiquinone, cytochrome b and cytochromes $c + c_1$ in TA3 ascites tumor cells. When NDGA was added, NAD(P)$^+$ was reduced, as indicated by the upward deflection of the 340–390 nm trace. NDGA also shifted the redox levels of ubiquinone and cytochrome b towards more oxidized states, as indicated by the upward deflection of the 275–245 nm trace and the downward deflection of the 430–410 nm trace, respectively. Moreover, the redox states of cytochromes $c + c_1$ were changed to more oxidized states. Similar results were observed with 786A ascites tumor cells (data not shown).

Thus, these results suggest not only that NDGA did not perceptibly inhibit electron flow from cytochrome b to oxygen, but also that the predominant inhibitory site of NDGA is located at some point before ubiquinone.

DISCUSSION

The effects of a very large number of phenolic compounds on the respiratory activity of mammalian

mitochrondia have been studied. Many phenols have been reported to uncouple the redox reactions from that of ADP phosphorylation [37,41,42]. Several other phenols have been shown to inhibit the mitochondrial electron flow at specific sites [41–43], whereas others have exhibited both uncoupling and inhibitory effects, which depend on the respiratory chain segment examined [44], or have no effect [43]. The biological action on mitochondrial respiration by phenolic compounds correlates with their physico-chemical properties, such as hydrophobicity, and electronic features [41, 42]. An uncoupling effect of phenols is predominant when the electronic coefficient is larger than that of the hydrophobic coefficient, whereas, if the electronic coefficient is smaller than that of the hydrophobic coefficient, an inhibitory effect is primarily observed [41, 45].

The results of this study suggest that NDGA, a highly hydrophobic polyhydroxyphenol, is an inhibitor of the reoxidation of NADH by the mitochondrial respiratory chain of TA3 and 786A ascites tumor cells, since oxygen consumption is similarly prevented by this compound, both in the presence and absence of CCCP; this, in turn, suggests that NDGA does not interfere with the phosphorylation mechanism or with the adenine nucleotide translocase (Fig. 4). These inhibition curves were sigmoidal in nature, thus indicating a cooperative inhibitory effect on the electron flow, which would suggest the existence of more than one site of inhibition. To elucidate its inhibitory kinetic mechanism, it would be necessary to perform experiments with at least isolated mitochondria.

Studies of the effect of NDGA on different segments of the respiratory chain demonstrated that the cytochrome oxidase activity was not affected (Fig. 5). Similarly, electron flow through the cytochrome b–c_1 complex was not inhibited, as indicated specifically by the failure of NDGA to inhibit the oxidation of ubiquinol (Fig. 6), suggesting that NDGA interferes with the components of the electron transfer system before ubiquinone. The partial inhibition of duroquinol oxidation observed in Fig. 5 might be explained by a steric effect arising at high concentrations of NDGA, preventing duroquinol from donating electrons to ubiquinone. This compound also caused a change of the redox state of $NAD(P)^+$ towards a more reduced level, as indicated by the upward deflection of the 340–390 nm trace (Fig. 6). This experiment and others determining the redox states of electron carriers (Fig. 6) suggest that NDGA interferes by inhibiting the energy-conserving site 1 of the respiratory chain, NADH dehydrogenase, excluding the interference of NDGA on both substrate transport systems and NAD^+-linked dehydrogenases. These findings suggest that the structural configuration of the dihydroxy moieties of NDGA is important for this reported biological activity, since only those lignans possessing a catechol moiety have exhibited an inhibitory effect on mitochondrial electron flow [24, 41]. Moreover, NDGA can be oxidized to give semiquinone radicals and further to form orthoquinones [2,46], competing with the ubiquinol/ubiquinone couple for their interaction sites in the respiratory chain.

ATP is required by living organisms to drive the majority, if not all, of their biosynthetic pathways, as well as for the maintenance of the intracellular ion balance and for specialized functions, such as muscle contraction and nerve transmission. It would seem that all tumor cells do not obtain the same proportion of their ATP from mitochondrial and glycolytic reactions. The slow and intermediate growth rate classes of tumor cells, as well as many normal tissues, obtain the bulk of their ATP from mitochondrial oxidative phosphorylation (more than 90% of the total), whereas the rapidly growing tumors, in the presence of high glucose concentrations, can obtain noticeable amounts of it from both sources, but with the higher proportion of ATP still being derived from the mitochondria (greater than 50% of the total [47]). Moreover, accumulated evidence indicates that oxidation of glutamine to CO_2, not glucose, is the major energy source for tumor cells, even in the presence of physiological levels of glucose [48–50]. On the other hand, hexokinase bound to mitochondria of highly glycolytic tumor cells, which is the catalyzer of the first reaction of glycolysis, has preferred access to mitochondrially generated ATP relative to cytosolic ATP [51]. Consequently, mitochondria from tumor cells would be the principal and the most important site of ATP synthesis.

NDGA caused a profound deflection in the electron flow through the NADH-CoQ segment, impairing the utilization of NAD^+-linked substrates, which would result in a pronounced decrease of ATP synthesis and an increase of NADH level. Thus, NDGA, besides inhibiting glucose absorption and glycolysis [1,5,27], would also act as an inhibitor of oxidative phosphorylation, preventing all ATP synthesis, which, in turn, would detain the cellular energy processes, triggering the complex chain of events that result in cell death. These findings would explain, in part, the antineoplastic activity of NDGA described by Burk and Woods [1].

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