Effects of Nordihydroguaiaretic Acid on the Chemiluminescence of Murine Phagocytes

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Abstract. Antioxidative effects of nordihydroguaiaretic acid (NDGA) on murine bone marrow phagocytes were studied using luminol- and lucigenin-amplified chemiluminescence (CL). NDGA applied *in vitro* strongly suppressed the opsonized zymosan particles stimulated CL response in a dose-dependent manner (in concentrations of 0.3–30 μg NDGA/ml), thus confirming its antioxidant activity. However, no effects were observed in mice and their serum samples when investigated one minute to one hour after an i.p. administration of NDGA. These differences suggest that NDGA may undergo a rapid metabolism *in vivo*.

Key words: Antioxidant — Nordihydroguaiaretic acid — Phagocyte — Chemiluminescence

Introduction

Nordihydroguaiaretic acid (NDGA), which occurs in the resinous exudates of many plants, is used as an antioxidant in fats and oils and shows a lot of other effects (Wang et al. 1991). It is known as a potent inhibitor of prostaglandin (PG) and leukotriene (LT) production via inhibiting cyclooxygenase and lipoxygenase metabolic pathways and thus may influence hemopoietic (Vore et al. 1989) and cancer cell (Snyder et al. 1989, Gati et al. 1990) proliferation as demonstrated *in vitro*. Moreover, it was shown that a decline in LT and PG production improves the inflammation response (Salmon and Garland 1991). Therefore, LT and PG inhibitors may act as potential therapeutic or protective agents.

Our recent studies have shown that NDGA administered to mice in a dose of 0.3 mg per mouse in a protective regimen one hour before gamma-irradiation stimulates postirradiation recovery of pluripotent and progenitor hemopoietic cell populations (Kozubík et al. 1993). Two mechanisms (acting separately or in combination) may be taken into account in the interpretation of these effects. First, NDGA may act positively on the regulatory processes controlling cell proliferation during the early phases of hemopoietic recovery. Second, NDGA could change

radiosensitivity of pluripotent hemopoietic cell populations by influencing the primary radiation-induced reaction, i.e. by scavenging of toxic reactive oxygen species (ROS).

It is clear that the generation of ROS also represents one of the main mechanisms by which phagocytes can mediate tissue injury (Dinerman and Mehta 1990). The adverse effects of ROS can be controlled by using antioxidants and antiinflammatory drugs (Dinerman and Mehta 1990, Lucchesi 1990). Therefore, activated phagocytes represent a good experimental system for studying antioxidative effects of these compounds. NDGA, a nonsteroidal compound with antiinflammatory effects, has not yet been tested in the whole organism concerning its antioxidative effects. In order to contribute to the understanding of NDGA action under these conditions we used a chemiluminescence (CL) method for the evaluation of the antioxidative effects of NDGA both *in vitro* and *in vivo*.

Materials and Methods

Animals: Male (CBA x C57Bl) F_1 hybrid conventional mice were kept under controlled light and temperature conditions; water and food were given ad libitum. The mice were subjected to the experimental procedure at the age of 12–14 weeks (28 ± 3 g of body weight).

Drug administration: NDGA (Aldrich-Chemie) was dissolved in ethanol, then diluted in an isotonic phosphate buffer (pH 7.4) and used in the following experimental systems:

a) NDGA solution (concentration range of 0.03–30 μg/ml) was added to a suspension of intact bone marrow cells *in vitro*.

b) NDGA (0.15–3.0 mg per mouse) was administered intraperitoneally in volumes of 0.2 ml to the mice one minute to 1 hour before serum sampling. 12 μl of serum was added to a suspension of intact bone marrow cells. Control animals were injected with the same volumes of the vehicle, i.e. ethanol and buffer. *In vivo* NDGA dosage was derived from effective concentrations as demonstrated before (Kozubík et al. 1993).

Chemiluminescence assay: The bone marrow of femoral diaphyses was flushed with a MEM medium (USOL Prague), the obtained cells were washed 3 times by MEM, and resuspended at a concentration of 1.5×10^7 cells/ml. Phagocytes in this cell suspension were activated by opsonized zymosan particles (OZP). The oxidative burst of the phagocytes was quantified by a chemiluminescence method, where luminol (Sigma) and lucigenin (Aldrich-Chemie) were used as the luminophores at a final concentration of 5×10^{-4} mol/l. It is generally accepted that lucigenin is specific for the superoxide anion, while luminol reacts with other oxygen species (Müller-Peddinghaus 1984, Bottu 1989). Kinetics of the luminol- and lucigenin-enhanced CL was measured for a period of 60 minutes using a Luminometer LKB Wallac 1251. The recorded parameters included the time and intensity of the peak activity (expressed in mV). Further details on the methods can be found in LKB Wallac Application Note 513.

Data evaluation: Each curve for the kinetics of the CL reaction was constructed by plotting 20 points, where each point represents a mean value obtained from 6 measurements. The

results (peak maxima and areas) were analyzed by the Student's t-test at significance levels of 5 and 1 percent.

Results

The chemiluminescence response values of activated phagocytes isolated from the bone marrow of untreated mice after the addition of NDGA *in vitro* in a concentration range of 0.03–30 μg/ml are referred-to in Table 1. In this experiment, NDGA significantly suppressed the oxidation of luminol at a concentration as low as 0.3 μg/ml; the higher NDGA concentrations produced especially strong effects. Lucigenin chemiluminescence was also suppressed, but the needed concentration of NDGA was 3 and 30 μg/ml. These effects demonstrate the antioxidative properties of NDGA in relation to phagocyte-generated ROS.

Table 1. Comparison of luminol- and lucigenin-enhanced chemiluminescence of activated phagocytes expressed as peak maxima and integral areas. The phagocytes were cultivated without NDGA (control) and after adding NDGA in a concentration range of 0.03–30 μg/ml.

Concentr. of NDGA (μg/ml)	Luminol		Lucigenin	
	Peak (mV)[a]	Integral[a]	Peak (mV)[a]	Integral[a]
Control	165.2 ± 14.3	473 261 ± 40 517	15.6 ± 1.1	45 376 ± 2 577
30	1.1 ± 0.05 $P \leq 0.01$	3 745 ± 29 $P \leq 0.01$	1.1 ± 0.03 $P \leq 0.01$	3 733 ± 33 $P \leq 0.01$
3	9.8 ± 1.1 $P \leq 0.01$	31 875 ± 4 296 $P \leq 0.01$	5.4 ± 1.2 $P \leq 0.01$	15 813 ± 3 305 $P \leq 0.01$
0.3	114.4 ± 11.9 $P \leq 0.05$	333 129 ± 35 394 $P \leq 0.05$	14.4 ± 0.9 –	43 575 ± 2 719 –
0.03	139.4 ± 13.0 –	414 401 ± 38 487 –	15.1 ± 1.0 –	46 463 ± 7 620 –

[a] Values represent the mean values ± S.E.M. ($n = 6$).

However, antioxidative properties of NDGA were not observed in those experiments where sera of mice pretreated with NDGA *in vivo* were tested. The serum from the experimental animals was collected 1 hour after administering 0.15–3.0 mg of NDGA per mouse and added immediately to the above described test system

instead of the NDGA. Neither luminol nor lucigenin oxidation differed from the reactions obtained with the sera of the corresponding control mice. Since NDGA is reported to have a short half-life (Lefer 1986), we also tested the sera of mice pretreated with the highest concentration of NDGA (i.e. 3 mg per mouse) given at shorter intervals before the serum collection (i.e. 1, 5, 15, and 30 minutes). No effects were observed in this case either (see Table 2).

Table 2. Effects of the sera of mice pretreated with 3 mg of NDGA (from 1 to 60 minutes before sera collection) on luminol- and lucigenin-enhanced chemiluminescence of intact mice phagocytes.

Time	Control		NDGA	
(min)	Peak (mV)[a]	Integral[a]	Peak (mV)[a]	Integral[a]
1	132.9 ± 12.6	325 905 ± 66 530	122.5 ± 4.2	288 585 ± 40 748
5	134.2 ± 8.7	309 707 ± 49 050	122.4 ± 8.1	293 760 ± 64 816
15	123.5 ± 3.4	299 772 ± 45 782	124.9 ± 2.9	297 489 ± 38 595
30	130.2 ± 7.4	311 297 ± 41 948	130.8 ± 12.5	310 565 ± 73 151
60	140.3 ± 52.1	325 703 ± 104 525	138.8 ± 29.2	337 011 ± 68 527

[a]Values represent the mean values ± S.E.M. ($n = 6$).

In the following experiments, the possible interference of serum factors with the effect of NDGA was studied. A suspension of phagocytes incubated with NDGA at concentrations of 0.3, 3.0 and 30.0 μg/ml reacted to opsonized zymosan with the same intensity regardless of whether the serum of intact mice was present or absent (Fig. 1).

Further experiments provided evidence that the 1-hour pretreatment of mice with NDGA administered intraperitoneally does not influence the CL activity of phagocytes in the bone marrow of these mice (data not shown).

Discussion

The present study demonstrates antioxidative effects of NDGA towards phagocyte-derived ROS *in vitro* in a dose-dependent manner. The different extent of suppression of luminol- and lucigenin-mediated CL at corresponding NDGA concentrations indicates that oxygen derivatives are directly removed by NDGA (Lefer 1986, Webb and Roth 1987).

Apart from this direct ROS removal, other mechanisms are likely to participate in curbing the CL activity. Other authors have also mentioned the importance of

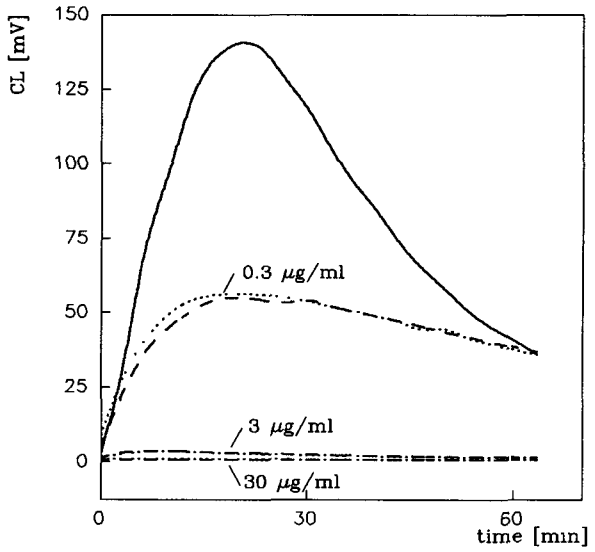

Figure 1. NDGA (concentrations of 0.3–30 μg/ml) effects on luminol-enhanced chemiluminescence in the absence or presence of the serum of intact mice. Dashed lines – samples without serum, dotted lines – serum samples, solid line – control.

antioxidants such as NDGA in providing important leads to the development of antiinflammatory compounds with multiple sites of action (Lefer 1986, Maloff et al. 1987, Henderson et al. 1989, Robison et al. 1990).

Our *in vitro* results are in good agreement with those of Webb and Roth (1987), who reported a lower amount of cytochrome c being reduced in the presence of 1–50 μg NDGA/ml (a measure of superoxide anion production) in a suspension of human neutrophils, and a suppression of the iodination reaction (a measure of the myeloperoxidase-hydrogen peroxide-halide activity). At the same time, the authors did not find any changes in the basic neutrophil functions (ingestion, random migration, antibody-dependent cell-mediated cytotoxicity) within this concentration range, i.e. these concentrations were not considered toxic to the cells.

On the other hand, the antioxidative properties of NDGA were not demonstrated *in vivo* under our experimental conditions. In addition, the evidence of not altered radiosensitivity (the same D_o values) of pluripotent (CFU-S) and committed (GM-CFC) hemopoietic cells of control mice and mice treated with NDGA *in vivo*, one hour before sublethal gamma-irradiation, support this conclusion (data not shown). These results indicate scepticism concerning the use of NDGA in goal-seeking influencing pathological processes with assumed increased ROS occurence. This may be due to the fact that NDGA, similarly to other lipoxygenase

inhibitors, has a very short half-life, thus making *in vivo* studies difficult to be performed (Lefer 1986). One of the causes of NDGA very short half-life could be the possibility of binding of the 5-lipoxygenase inhibitors to plasma proteins which could compromise their activity (Salmon and Garland 1991). A series of our experiments designed to detect possible interference of serum factors in the effects of NDGA excluded this type of effects.

However, our previous studies indicate that difficulties mentioned above are not the reason for the rejecting of NDGA use under *in vivo* conditions. Compared with other nonsteroidal antiinflammatory drugs, NDGA is less toxic (Webb and Roth 1987) and as an inhibitor of PGs and LTs generation it can influence positively processes of regulation of damaged hemopoietic tissue cell proliferation *in vivo* (Kozubík et al. 1993).

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Final version accepted June 25, 1993