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Yong Soo Lee · Robert D. Wurster

Mechanism of potentiation of LY83583-induced growth inhibition by sodium nitroprusside in human brain tumor cells

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Abstract The effect of 6-anilino-5,8-quinolinedione (LY83583), an inhibitor of guanylyl cyclase (GC), on the growth of human brain tumor cells (U-373 MG astrocytoma and SK-N-MC neuroblastoma) was evaluated. LY83583 inhibited the growth of these cells in a dose-dependent manner. This growth inhibition was found to be the result of decreased cell viability as assessed by the trypan blue exclusion method. The LY83583-induced decrease in cell viability was not altered by dibutyryl cyclic GMP, but significantly was reversed by superoxide dismutase and catalase, indicating that these effects of LY83583 may not be due to the inhibition of GC, but due to the formation of superoxide anion. The LY83583-induced decrease in cell viability was potentiated by cotreatment with sodium nitroprusside (SNP), a nitric oxide (NO) donor. This SNP-induced potentiation was significantly blocked by various scavengers for hydroxyl radicals or by intracellular Ca^{2+} release blockers. These results suggest that the potentiation effects of SNP may be mediated through the generation of hydroxyl radicals which can be formed by the interaction of superoxide anion (from LY83583) and NO (from SNP), and that intracellular Ca^{2+} release from internal stores may play an important role in the cytotoxic mechanism of hydroxyl radicals.

Key words LY83583 · Sodium nitroprusside · Human brain tumors · Nitric oxide

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Yong Soo Lee
Department of Neurological Surgery, Loyola University Medical Center, Maywood, IL 60153, USA

R. D. Wurster (✉)
Department of Physiology, Loyola University Medical Center, 2160 S. First Ave., Maywood, IL 60153, USA

Introduction

Oxygen free radicals (OFRs) have been implicated in carcinogenic processes induced by radiation and some chemical carcinogens [3, 20]. Paradoxically, OFRs mediate a very important part of the mechanism of antitumor agents [13].

LY83583 (6-anilino-5,8-quinolinedione) has been shown to lower the tissue content of cyclic GMP in various organs [17], and to inhibit endothelium-dependent vasorelaxation [10]. Although the exact mechanisms are unknown, it has been suggested that OFRs mediate these actions of LY83583 [6].

We have previously reported that sodium nitroprusside (SNP) inhibits the growth of human brain tumor cells and that nitric oxide (NO) is responsible for this action of SNP [8]. Recent studies have demonstrated that NO can interact with superoxide anion yielding the peroxynitrite anion which decays rapidly to form the hydroxyl radical, the most toxic species of OFR [9].

Intracellular Ca^{2+} appears to be an important factor in the processes of cell proliferation [18] and cell death [12]. OFRs have been shown to modulate intracellular Ca^{2+} signalling mechanisms [2, 5, 15].

Thus, in this study we investigated the effect of LY83583 and the interactions of LY83583 and SNP on the control of the growth of human brain tumor cells and explored the mechanism of these interactions relating to intracellular Ca^{2+} signals, using U-373 MG human astrocytoma and SK-N-MC human neuroblastoma cell lines as model cellular systems.

Materials and methods

Materials

U-373 MG human astrocytoma and SK-N-MC human neuroblastoma cell lines were purchased from American Type Culture Collection (Rockville, Mass). The powders for Eagle's minimum essential

medium (MEM) and Earle's basal salt solution (EBSS), trypsin solution, trypan blue, sodium pyruvate, dantrolene (DANT), ruthenium red (RR), N^2,2'-*O*-dibutyryl guanosine 3',5'-cyclic monophosphate (db cyclic GMP), superoxide dismutase (SOD), catalase (CT), mannitol (MT), thiourea (TU), 1,3-dimethylthiourea (DMTU) and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, Mo.). LY83583 was from Calbiochem (La Jolla, Calif.). 3,4,5-Trimethoxybenzoic acid-8-(diethylamino)octyl ester (TMB) was from Aldrich Chemical Co. (Milwaukee, Wis). Fetal bovine serum (FBS) and antibiotic mixture (penicillin and streptomycin) were purchased from GIBCO (Grand Island, N.Y.). LY 83583 was dissolved in ethanol.

Cell culture.

Cells were grown at 37°C in an incubator in a humidified atmosphere of 5% CO_2/95% air in MEM supplemented with 10% FBS, 200 IU/ml penicillin, 200 µg/ml streptomycin and 1 m*M* sodium pyruvate. The culture medium was replaced every other day. After attaining confluency the cells were subcultured by trypsinization.

Cell growth studies.

Cells from 4–5-day-old cultures were seeded in equal numbers in 35×10 mm culture dishes at a density of 1.5×10^5 and 2×10^5 cells/dish for the astrocytoma and neuroblastoma cell lines, respectively. The volume of the medium in the dishes was 2 ml. The drugs to be tested were added to the cultures 1 day after seeding in order to ensure uniform attachment of the cells at the start of the experiments. The cells were grown for an additional 2 days. Drugs and culture medium were replaced every day. In control experiments cells were grown in the same medium containing drug-free vehicle. Cell growth was assessed by counting viable cells in a hemocytometer using trypan blue exclusion after trypsinization.

Data analysis.

All experiments were performed a minimum of four times. All data are displayed as the percentage of the control condition which was taken as 100%. Data are expressed as mean ± standard error of the mean (SEM) and were analyzed using one way analysis of variance (ANOVA) and Student-Newman-Keul's test for individual comparisons. *P*-values less than 0.05 were considered to be statistically significant.

Results

LY83583 inhibited the growth of both human astrocytoma and neuroblastoma cells in a dose-dependent manner as shown in Fig. 1. This growth inhibition was the result of a decrease in cell viability as evaluated by the trypan blue exclusion method. The response of astrocytoma cells to LY83583 was more sensitive than that of neuroblastoma cells. The LY83583-induced inhibition of the growth of both tumor cell lines was potentiated by 200 µ*M* SNP (Fig. 1). This concentration of SNP alone did not significantly alter tumor cell growth.

Since LY83583, a known potent inhibitor of soluble guanylyl cyclase (GC), may decrease the intracellular level of cyclic GMP, the effect of db cyclic GMP, a cell membrane-permeable analogue of cyclic GMP, on

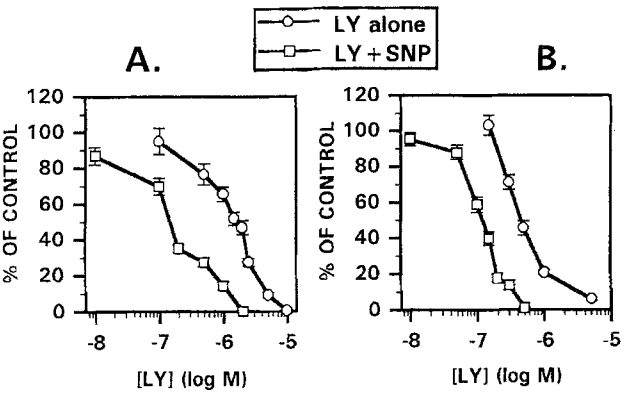

Fig. 1A, B Potentiation of LY83583-induced growth inhibition by 200 µ*M* SNP in SK-N-MC human neuroblastoma cells (**A**) and U-373 MG human astrocytoma cells (**B**). The numbers of viable cells (percent of control) with SNP (200 µ*M*) alone were 100.35 ± 6.24 and 90.33 ± 3.03% in the SK-N-MC and U-373 MG cell lines, respectively. The results are expressed as the percentage change in the number of viable cells in relation to that obtained in the drug-free vehicle. The data points represent the mean ± SEM

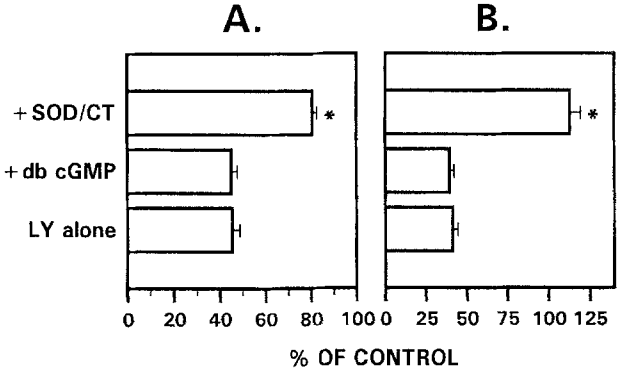

Fig. 2A, B LY83583-induced growth inhibition is mediated not by a decrease in cyclic GMP, but by the formation of free radicals in SK-N-MC human neuroblastoma cells (**A**) and U-373 MG human astrocytoma cells (**B**). The concentrations of LY83583 used in these experiments were 2 and 0.5 µ*M* for the SK-N-MC and U-373 MG cell lines, respectively. SOD (100 U/ml), CT (100 U/ml) and db cyclic GMP (1 m*M*) were used in these experiments. Results are expressed as the percentage change in the number of viable cells in relation to that obtained in the drug-free vehicle. The columns represent the mean ± SEM (*$P < 0.05$ compared with LY 83583 alone)

LY83583-induced inhibition of the growth of these tumor cell lines was examined. As shown in Fig. 2, db cyclic GMP did not alter the effects of LY83583 in either cell line. However, treatment with superoxide dismutase (SOD) and catalase (CT), scavengers of superoxide anion and hydrogen peroxide, respectively, significantly abolished the growth-inhibitory effects of LY83583 in both cell lines.

SNP-induced potentiation was significantly reversed by various hydroxyl radical scavangers as shown in Fig. 3 A, B. TU, DMTU, MT and DMSO which have been used as hydroxyl radical scavengers in a variety of

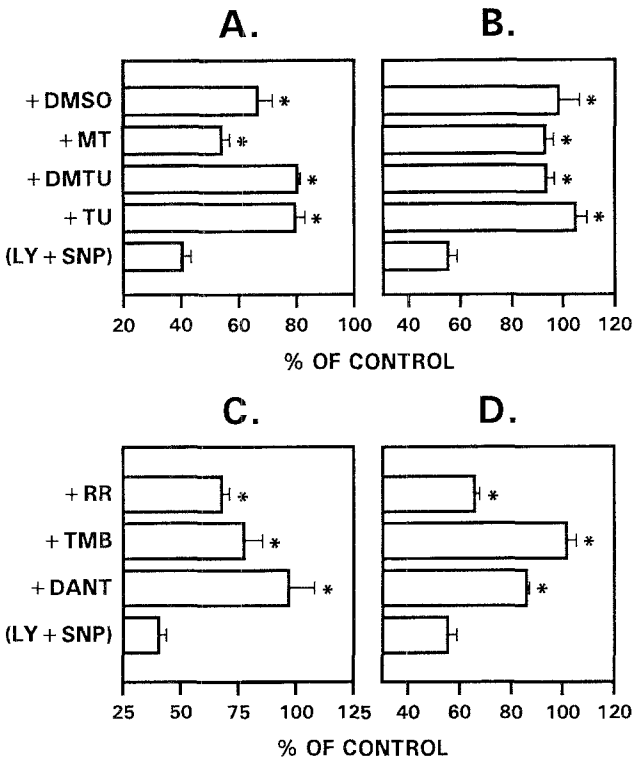

Fig. 3A–D The mechanism of the SNP-induced potentiation in SK-N-MC human neuroblastoma cells (**A** and **C**) and U-373 MG human astrocytoma cells (**B** and **D**). The generation of hydroxyl radicals mediates the actions of LY83583 and SNP (**A** and **B**). Intracellular Ca^{2+} release blockers also effectively reverse these actions (**C** and **D**). The concentrations of LY83583 used in these experiments were 0.15 and 0.1 μM for the SK-N-MC and U-373 MG cell lines, respectively. SNP (200 μM), TU (10 mM), DMTU (10 mM), MT (20 mM), DMSO (0.5%), DANT (25 μM), TMB (10 μM) and RR (1 μM) were used. Results are expressed as the percentage change in the number of viable cells in relation to that obtained in the drug-free vehicle. The columns represent the mean ± SEM (*$P < 0.05$ compared with LY 83583 + SNP)

cells [19], were used in these experiments. DANT and TMB, known inhibitors of intracellular Ca^{2+} release [16, 21] also significantly blocked the potentiation effects of SNP (Fig. 3C, D). RR, another inhibitor of intracellular Ca^{2+} release, specifically, from the ryanodine-sensitive Ca^{2+} pools [1], showed the same results as DANT and TMB (Fig. 3 C, D). The concentrations of these drugs were chosen such that they did not significantly affect the growth of the tumor cells.

Discussion

This study clearly showed that LY83583 inhibits the growth of human brain tumor cells in a dose-dependent manner (Fig. 1). The growth inhibition induced by LY83583 was found to be due to a decrease in cell viability. Considering the well-known action of LY83583 on the level of intracellular cyclic GMP [17], the LY83583-induced decrease in cell viability may be the result of a decreased level of cyclic GMP. However, the results showed that the effects of LY83583 were not altered by cotreatment with db cyclic GMP, but significantly reversed by SOD and CT (Fig. 2), suggesting that LY83583 may act through the generation of free radicals, particularly superoxide anion. The generation of superoxide anion by LY83583 has been previously reported in other cellular systems [11]. Moreover, db cyclic GMP alone (up to 2 mM) did not induce a significant alteration in the growth of either tumor cell line (data not shown), which further implies that the cyclic GMP signalling pathway may not play an important role in the growth of these tumor cells.

In order to further examine the possible involvement of the cyclic GMP pathway in the actions of LY83583, we tested the effects of SNP, which has an opposite action in terms of regulating cyclic GMP levels, on the LY83583-induced inhibition of these tumor cell lines. SNP did not inhibit, and indeed potentiated, the actions of LY 83583 (Fig. 1). These results further demonstrate that the actions of LY83583 are not associated with the cyclic GMP pathway.

The next part of our study sought to investigate the mechanism underlying the SNP-induced potentiation of the actions of LY83583. Previously, we have found that SNP inhibits the growth of human brain tumor cells through the release of NO [8]. NO has been shown to interact with the superoxide anion and to produce peroxynitrite which is eventually broken down to hydroxyl radical [9]. Since the results of this study showed that the actions of LY83583 may be through the generation of superoxide anion, the SNP-induced potentiation can be hypothesized to be a result of the formation of hydroxyl radicals. In order to test this hypothesis, we examined the effects of various hydroxyl radical scavengers on the potentiation action of SNP. All the scavengers (TU, DMTU, MT and DMSO) were found to effectively block the SNP-induced potentiation of the actions of LY83583 (Fig. 3 A, B). These results suggest that SNP can generate NO which interacts with superoxide anions (from LY83538), then forming peroxynitrite, and ultimately leading to the formation of highly toxic hydroxyl radicals.

The cytotoxic action of OFRs, including hydroxyl radicals, is well established [14]. Free radical-induced cytotoxicity has been shown to be mediated by their interactions with virtually all cellular macromolecules [20]. Recently, it has been suggested that intracellular Ca^{2+} signals are involved in the mechanisms of the free radical actions [2, 5]. Thus, in order to test the possible involvement of intracellular Ca^{2+} signalling mechanisms in the interactions of SNP and LY83583 observed in this study, we examined the effects of several intracellular Ca^{2+} release blockers on their actions. The results showed that DANT, TMB and RR significantly reversed the effects of LY83583 and SNP in both cell lines (Fig. 3 C, D) These results suggest that

intracellular Ca^{2+} release from internal stores may be triggered by hydroxyl radicals (from the interactions of LY83583 and SNP), which may lead to the observed decrease in cell viability. The prolonged increase in intracellular free Ca^{2+} concentration has been shown to be a common mechanism of cell death [12]. Recent observations have shown that Ca^{2+} release from internal stores is enough to trigger apoptosis, a naturally occurring cell death mechanism [4], and that blocking this release can also effectively inhibit induced cell death [7]. Thus, the results of this study further suggest that increased intracellular Ca^{2+} may play an important role in the free radical-induced cell death mechanism.

In conclusion, LY83583 decreased brain tumor cell viability through the formation of free radicals, and SNP further potentiated these actions of LY83583 by the generation of more toxic hydroxyl radicals. In addition, the cytotoxic actions of hydroxyl radicals may be mediated by increased intracellular Ca^{2+} concentrations. These results suggest that a combination of LY83583 and SNP may be a valuable strategy in the therapy of human brain tumors.

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