Intracellular Ca^{2+} Mediates Lipoxygenase-induced Proliferation of U-373 MG Human Astrocytoma Cells

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The role of intracellular Ca^{2+} in the regulation of tumor cell proliferation by products of arachidonic acid (AA) metabolism was investigated using U-373 MG human astrocytoma cells. Treatment with nordihydroguaiaretic acid (NDGA), a lipoxygenase (LOX) inhibitor, or caffeic acid (CA), a specific 5-LOX inhibitor, suppressed proliferation of the tumor cells in a dose-dependent manner. However, indomethacin (Indo), a cyclooxygenase (COX) inhibitor, did not significantly alter proliferation of the tumor cells. At anti-proliferative concentrations, NDGA and CA significantly inhibited intracellular Ca^{2+} release induced by carbachol, a known intracellular Ca^{2+} agonist in the tumor cells. Exogenous administration of leukotriene B_4 (LTB_4), an AA metabolite of LOX pathway, enhanced proliferation of the tumor cells in a concentration-dependent fashion. In addition, LTB_4 induced intracellular Ca^{2+} release. Intracellular Ca^{2+} inhibitors, such as an intracellular Ca^{2+} chelator (BAPTA) and intracellular Ca^{2+}-release inhibitors (dantrolene and TMB-8), significantly blocked the LTB_4-induced enhancement of cell proliferation and intracellular Ca^{2+} release. These results suggest that LOX activity may be critical for cell proliferation of the human astrocytoma cells and that intracellular Ca^{2+} may play a major role in the mechanism of action of LOX.

Key words : Human astrocytoma cells, Cell proliferation, Lipoxygenase, Intracellular Ca^{2+}

INTRODUCTION

Arachidonic acid (AA) is metabolized via cyclooxygenase (COX) and lipoxygenase (LOX) to produce prostaglandins and leukotrienes, respectively. These eicosanoids which have been detected in many different tissues including human brain tumors (Castelli *et al.*, 1987), are known to act as second messengers that can alter many ongoing cellular processes (Di Marzo, 1995; Harder *et al.*, 1997). In particular, these AA metabolites appear to have an effect on cell proliferation (Hashimoto *et al.*, 1997; Schror and Weber, 1997). However, the effect of AA metabolites on cell proliferation is not consistent. They act as positive (Bortuzzo *et al.*, 1996) or negative (Ren and Dziak, 1991) regulators of cell proliferation.

Accumulating evidence implicates that intracellular Ca^{2+} is an important regulator of cell proliferation (Villereal and Byron, 1992). Previously, we have also reported that intracellular Ca^{2+} plays a key role in the regulatory effects of many drugs on proliferation of human brain tumor cells (Lee *et al.*, 1994; Lee *et al.*, 1995). Recent findings indicate that AA metabolites have an influence on intracellular Ca^{2+}-regulating mechanisms in a variety of cell types (Alonso-Torre and Garcia-Sancho, 1997; Striggow and Ehrlich, 1997).

Although Wilson *et al.* (1989) have reported that nordihydroguaiaretic acid (NDGA), a LOX inhibitor, suppresses proliferation of human glioma cells, the role of AA metabolites in the proliferation of human brain tumor cells has not been clearly decided yet.

In this study we investigated the effects of AA metabolites on the proliferation of human brain tumor cells and elucidated the mechanism of action of AA metabolites relating to intracellular Ca^{2+}, using U-373 MG human astrocytoma cells as a model cellular system.

MATERIALS AND METHODS

Materials

U-373 MG human astrocytoma cell line was purchased from American Type Culture Collection (Rockville, MA). The powders Eagle's minimum essential medium (MEM) and Earle's basal salt solution (EBSS), trypsin solution, trypan blue, sodium pyruvate, ethylene glycol-bis-(aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), indomethacin (Indo), caffeic acid (CA), nordihydroguaiaretic acid (NDGA), leukotriene B_4 (LTB_4), dantrolene

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(Dant), 3,4,5-trimethoxybenzoic acid-8-(diethylamino)-octyl ester (TMB-8), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), carbachol (Carb) and all salt powders were obtained from Sigma Chemical CO. (St. Louis, MO). [^{3}H]thymidine was supplied by Amersham (Arlington, IL). 1-(2,5-Carboxyoxazol-2-yl-6-aminobenzfuran-5-oxyl)-2-(2'-amino-methylphenoxy)-ethane-N,N,N'N'-tetraacetoxylmethyl ester (Fura-2) and bis-(*o*-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid/acetoxymethyl ester (BAPTA) were from Molecular Probes, Inc. (Eugene, OR). Fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). Indo, NDGA, CA and LTB_4 were prepared as stock solutions in absolute ethanol, then diluted with aqueous medium to the final desired concentrations. The stock solution of LTB_4 was stored at -20°C and diluted immediately prior to the initiation of the experiments. The stock solution of drugs was sterilized by filtration through 0.2 μm disc filters (Gelman Sciences: Ann Arbor, MI).

Cell culture

Cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in a MEM supplemented with 10% FBS, 200 IU/ml penicillin, 200 μg/ml of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After attaining confluence the cells were sub-cultured following trypsinization.

Cell proliferation assay

Cell proliferation was assessed by [^{3}H]thymidine incorporation assay. Cells from 4-5-day old cultures were incubated in 96-well plates at an initial density of 10^4 cells/well. Drugs to be tested were added to cultures 1 day after seeding to ensure uniform attachment of cells at the onset of the experiments. The cells were grown for an additional 2 days. Drugs and culture medium were replaced everyday. In control experiments cells were grown in the same media containing drug-free vehicle. After a period of incubation, 1 μCi/well of [^{3}H]thymidine was added and cells were incubated for a further 18 hr. After cells were harvested onto a glass fiber filter, [^{3}H]thymidine incorporation was measured with a β-scintillation counter (Pharmacia, Uppsala, Sweden). Relative proliferation of drug-treated cells was obtained by percent change of CPM compared to control cells.

Intracellular Ca^{2+} measurement

Aliquots of the tumor cells, cultured for 3~5 days, were washed in EBSS. Then, 2 μM Fura-2 was added, and the cells were incubated for 60 min at room temperature (22~23°C). Unloaded Fura-2 was removed by centrifugation at 150×g for 3 min. Cells were resuspended at a density of 2×10^6 cells/ml in Ca^{2+}-free Krebs-Ringer buffer (KRB) containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM HEPES and 6 mM glucose (pH 7.4), transferred to a quartz cuvette and stirred continuously. Fluorescence emission (510 nm) was monitored with the excitation wavelength cycling between 340 and 380 nm at 37°C using a Hitachi F4500 fluorescence spectrophotometer. At the end of an experiment, fluorescence maximum and minimum values at each excitation wavelength were obtained by firstly permeabilizing the cells with 0.1% Triton X-100 (maximum) and then adding 10 mM EGTA (minimum). With the maximum and minimum values, the 340:380 nm fluorescence ratio was converted into free Ca^{2+} concentration using Fura-2 Ca^{2+}-binding constant (224 nM) and the formula described by Grynkiewicz *et al.* (1985).

Data analysis

All experiments were performed four times. All data were displayed as % of control condition. Data were 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 are considered statistically significant.

RESULTS

Effects of AA metabolism inhibitors on cell proliferation

To examine the role of AA metabolism via the COX and LOX pathways in the proliferation of human astrocytoma cells, we tested the effects of COX and LOX inhibitors on the proliferation of U-373 MG human astrocytoma cells. In these experiments, Indo was used as a COX inhibitor (Ferreira *et al.*, 1971), and NDGA and CA were used as LOX inhibitors (Koshihara *et al.*, 1984; Salari *et al.*, 1984). Indo did not alter proliferation of the tumor cells as shown in Fig. 1. However, NDGA and CA suppressed proliferation of the tumor cells in a dose-dependent manner as shown in Fig. 1. The concentrations of half-maximum effects (EC_{50}) of NDGA and CA were about 5 and 10 μM, respectively.

Effects of LOX inhibitors on Carb-induced intracellular Ca^{2+} release

To examine the relationship between the observed anti-proliferative actions of LOX inhibitors and intracellular Ca^{2+} signals, we tested the effects of these LOX inhibitors on the increased intracellular Ca^{2+} concentration induced by Carb, a muscarinic receptor agonist, which acts as a mitogen (Larocca and Almazan,

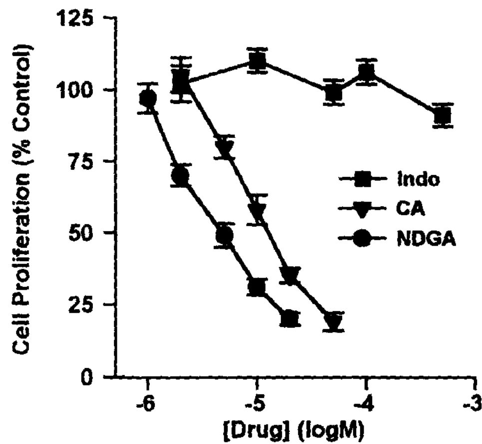

Fig. 1. Effects of Indo, a COX inhibitor, and NDGA and CA, LOX inhibitors, on cell proliferation of U-373 MG human astrocytoma cells. Cell proliferation assay was done by [^{3}H]-thymidine incorporation method. Results are expressed as percent change of control condition in which cells were grown in medium containing drug-free vehicle. Data points represent the mean values of four replications with bars indicating SEM.

1997). Our previous study showed that extracellular Ca^{2+} influx is not involved in both the Carb-induced increased intracellular Ca^{2+} concentration and cell proliferation in the tumor cells used in the present study (Lee *et al.*, 1993a; Lee *et al.*, 1993b). Thus, we used a Ca^{2+}-free buffer solution for intracellular Ca^{2+} measurement. As shown in Fig. 2A, 0.8 mM of Carb induced intracellular Ca^{2+} release, which is consistent with previous reports (Lee *et al.*, 1993a; Lee *et al.*, 1993b). NDGA (5 μM) and CA (10 μM) significantly inhibited the Carb-induced intracellular Ca^{2+} release at the anti-proliferative concentrations as depicted in Fig. 2A and 2B.

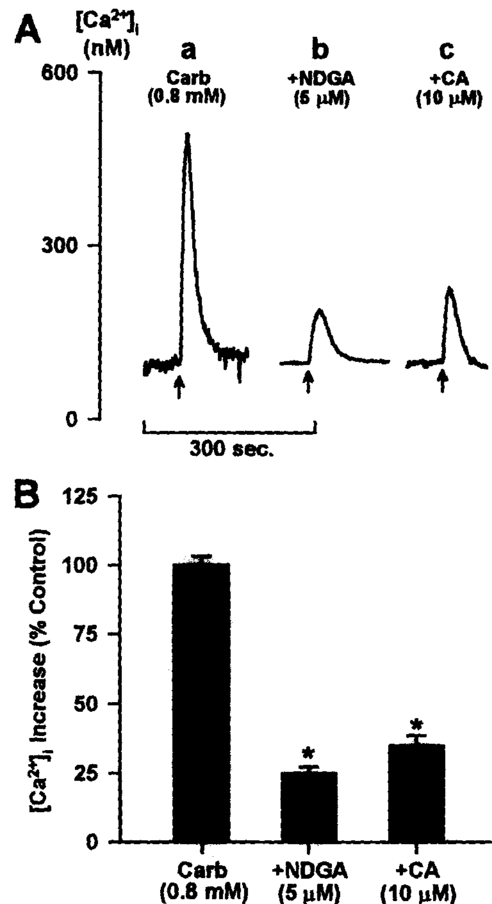

Fig. 2. Effects of LOX inhibitors on Carb (0.8 mM)-induced intracellular Ca^{2+} release in U-373 MG human astrocytoma cells. Aliquots of 2×10^6 cells/ml were incubated with 2 μM Fura-2 for 60 min at room temperature (22~23°C). The cells were washed, resuspended in Ca^{2+}-free buffer solution, and transferred to a quartz cuvet for fluorescence measurements. The data (A) represent intracellular Ca^{2+} changes with time. Arrows show the time points for addition of 0.8 mM of Carb. In these experiments 5 μM NDGA (b) or 10 μM CA (c) were applied 3 min before fluorescence measurements. Quantitative changes (B) were expressed as percent changes of the increased intracellular Ca^{2+} concentration induced by the drug compared to Carb alone. Each column represents the mean value of four replications with bars indicating SEM (*$p<0.05$ compared to Carb alone).

Effects of Ca^{2+} inhibitors on LTB_4-induced enhancement of cell proliferation

The effects of LTB_4, a LOX metabolite of AA (Stjernschantz, 1984), on the proliferation of the tumor cells were studied, and the results are depicted in Fig. 3A. LTB_4 induced a dose-dependent enhancement of proliferation of the tumor cells. The maximum enhancement of cell proliferation by LTB_4 was about 160% compared to control condition. EC_{50} value of the proliferation-enhancing activity of LTB_4 was about 10 nM. To elucidate the mechanism of this action of LTB_4, the effects of intracellular Ca^{2+} inhibitors on the LTB_4-induced proliferation enhancement were examined. In these experiments BAPTA, an intracellular Ca^{2+} chelator (Jiang *et al.*, 1994), Dant and TMB-8, intracellular Ca^{2+} release blockers (Rittenhouse-Simmons and Deykin, 1978; Zhang and Melvin, 1993) were used as Ca^{2+} inhibitors. Fig. 3B shows that treatment with either 1 μM BAPTA, 20 μM Dant or 5 μM TMB-8 significantly suppressed the proliferation enhancement induced by 0.1 μM LTB_4. The concentrations of these intracellular Ca^{2+} inhibitors used in these experiments were chosen at which they did not significantly alter cell proliferation.

Effects of Ca^{2+} inhibitors on LTB_4-induced intracellular Ca^{2+} release

To clarify the major role of intracellular Ca^{2+} as a signal transducer in the proliferation-enhancing action of LTB_4, we tested whether LTB_4 can alter intracellular Ca^{2+} concentration. LTB_4 induced increased intracellular

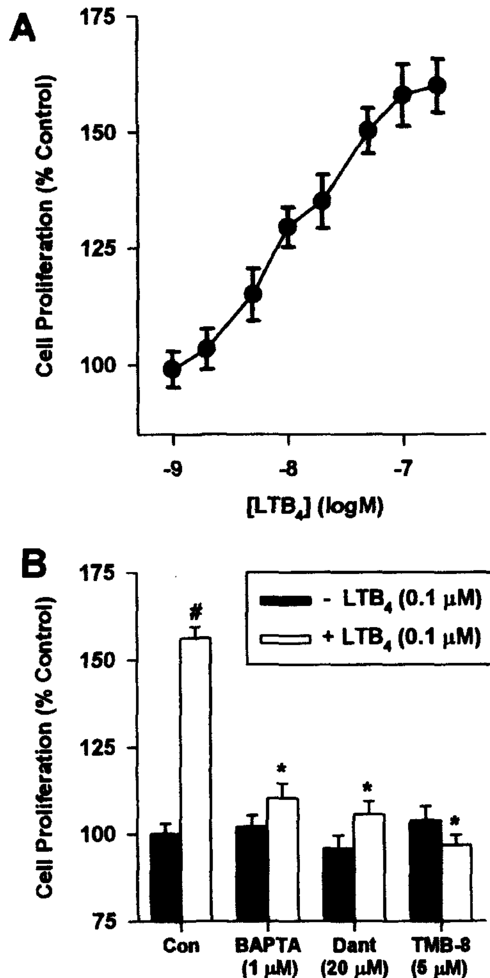

Fig. 3. Effects of Ca^{2+} inhibitors on LTB_4-induced enhancement of cell proliferation in U-373 MG human astrocytoma cells. Cell proliferation assay was done by $[^3H]$-thmidine incorporation method. Results are expressed as percent change of control condition in which cells were grown in medium containing drug-free vehicle. Data points (A) or columns (B) represent the mean values of four replications with bars indicating SEM (#$p<0.05$ compared to control; *$p<0.05$ compared to LTB_4 alone).

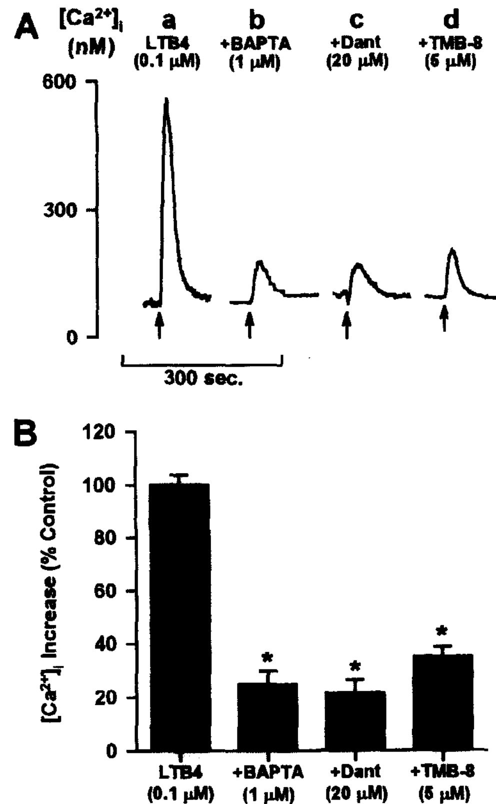

Fig. 4. Effects of Ca^{2+} inhibitors on LTB_4 (0.1 μM)-induced intracellular Ca^{2+} release in U-373 MG human astrocytoma cells. Aliquots of 2×10^6 cells/ml were incubated with 2 μM Fura-2 for 60 min at room temperature (22~23°C). The cells were washed, resuspended in Ca^{2+}-free buffer solution, and transferred to a quartz cuvet for fluorescence measurements. The data (A) represent intracellular Ca^{2+} changes with time. Arrows show the time points for addition of 0.1 μM LTB_4. In these experiments 1 μM BAPTA, (b), 20 μM Dant (c) or 5 μM TMB-8 (d) were applied 5 min before fluorescence measurements. Quantitative changes (B) were expressed as percent changes of the increased intracellular Ca^{2+} concentration induced by the drug compared to LTB_4 alone. Each column represents the mean value of four replications with bars indicating SEM (*$p<0.05$ compared to LTB_4 alone).

Ca^{2+} concentration as shown in Fig. 4A. The intracellular Ca^{2+} increase by LTB_4 was not dependent on the presence of Ca^{2+} in the buffer solution, indicating that LTB_4 induces only internal Ca^{2+} release (data not shown). Pre-treatment with either 1 μM BAPTA, 20 μM Dant or 5 μM TMB-8 significantly suppressed the LTB_4-induced intracellular Ca^{2+} release as shown in Fig. 4A and 4B.

DISCUSSION

The results of the present study show that AA metabolites of the LOX pathway are critical for the proliferation of human astrocytoma cells. The following observations give evidence on the growth-regulatory action of LOX in the tumor cells. The inhibition of LOX by NDGA or CA reduced proliferation of the tumor cells (Fig. 1). Exogenous administration of LTB_4, a product of LOX pathway, did stimulate proliferation of the tumor cells (Fig. 2A).

Recent reports indicate that prostaglandins, products of AA metabolism via COX pathway, have a regulatory role in cell proliferation (Hashimoto *et al.*, 1997; Schror and Weber, 1997). However, in the present study we found no effects of Indo, a COX inhibitor, on the proliferation of the human astrocytoma cells (Fig. 1), suggesting that COX activity may not be important for the growth regulation in the tumor cells.

The exact mechanism of action of LOX on the proliferation of the human astrocytoma cells is not known. However, the results of the present study suggest that intracellular Ca^{2+} may mediate the LOX-

induced proliferation regulation in the tumor cells (Figs. 2, 3 and 4). Pre-treatment with LOX inhibitors, NDGA and CA, significantly inhibited agonist (Carb)-induced intracellular Ca^{2+} release at anti-proliferative concentrations (Fig. 2). Intracellular Ca^{2+} inhibitors, such as intracellular Ca^{2+} release blockers (Dant and TMB-8) and an intracellular Ca^{2+} chelator (BAPTA) significantly suppressed the LTB_4-induced enhancement of cell proliferation (Fig. 3B). LTB_4 itself induced intracellular Ca^{2+} release and this effect of LTB_4 was significantly suppressed by the pre-treatment with these intracellular Ca^{2+} inhibitors (Fig. 4). These results strongly suggest that LOX metabolites of AA may evoke the activation of intracellular Ca^{2+} signals, leading to proliferation of the tumor cells.

In contrast with the results of the present study, Anderson *et al.* (1996) have reported that MK886, a 5-LOX inhibitor, increased intracellular Ca^{2+} concentration in U937 monoblastoid cells. They have also found that MK886 inhibited cell proliferation and induced apoptosis of the cells (Anderson *et al.*, 1996). Considering the intracellular Ca^{2+} increase is believed to play an important role in the mechanism of apoptosis (McConkey and Orrenius, 1997), the effect of MK886 on the intracellular Ca^{2+} concentration may be closely related with its action of apoptosis rather than that of anti-proliferation. Nevertheless, the differential effects of these LOX inhibitors on the intracellular Ca^{2+} concentration between these two systems remain to be further characterized and determined.

Intracellular Ca^{2+} appears to play an important role in cell proliferation (Metcalfe *et al.*, 1986; Whitfield *et al.*, 1987; Geck and Bereiter-Hahn, 1991). Previously, we have also shown that increased intracellular Ca^{2+} concentration is necessary for cell proliferation in human astrocytoma cells (Lee *et al.*, 1993b; Lee *et al.*, 1994). However, the source of increased intracellular Ca^{2+} is controversy, and intracellular Ca^{2+} release and/or extracellular Ca^{2+} influx, depending on the cell types and mitogens (Lee *et al.*, 1993b; Lee *et al.*, 1994; Keller *et al.*, 1997; Yoo *et al.*, 1997). LTB_4 which is shown to act as a mitogen for the human astrocytoma cells in the present study, appears to increase intracellular Ca^{2+} concentration in many cell types (Goldman *et al.*, 1985; Owman *et al.*, 1997; Striggow *et al.*, 1997). The source of LTB_4-induced increased intracellular Ca^{2+} is not uniform in different cell types, and either intracellular Ca^{2+} release (Montero *et al.*, 1994; Striggow *et al.*, 1997), extracellular Ca^{2+} influx (Owman *et al.*, 1997), or both (Lew *et al.*, 1984). In the human astrocytoma cells used in the present study, LTB_4 induced intracellular Ca^{2+} release without extracellular Ca^{2+} influx (Fig. 4A). We speculate that this discrepancy in the source of the LTB_4-induced intracellular Ca^{2+} rise may be due to different signaling pathways of LTB_4 in different cell types.

Although the results of the present study give strong evidence that intracellular Ca^{2+} signals act as a major mediator for the LTB_4-induced cell proliferation, other mechanisms may possibly be involved. LTB_4 has been shown to induce D-myo-inositol-1,4,5-trisphosphate ($InsP_3$) accumulation and inhibition of adenylyl cyclase (Yokomizo *et al.*, 1997). LTB_4 also promotes the rate of H_2O_2 generation through activation of protein kinase C (PKC) (Perkins *et al.*, 1995). Since these events are well known to be actively involved in the signaling mechanisms of cell proliferation (Berridge *et al.*, 1985; Burdon, 1996; Caponigro *et al.*, 1997), they may play a role in the proliferative action of LTB_4.

In conclusion, AA metabolites of LOX pathway regulate proliferation of U-373 MG human astrocytoma cells. Intracellular Ca^{2+} may mediate the LTB_4-induced cell proliferation. These results suggest that LOX and intracellular Ca^{2+} signaling pathways may be good chemotherapeutic targets for human astrocytomas.

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