Effects of Trapidil and Suramin on Growth Factor-induced Calcium Response and Tyrosine Phosphorylation in Human Glioma Cells

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Abstract

Platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) induce the proliferation of glioma cells in vitro. Trapidil and suramin inhibit this growth factor-stimulated glioma cell growth, but the mechanisms are not fully understood. The effects of trapidil and suramin on PDGF- and EGF-induced early biochemical events in T98G cells were studied. PDGF induced a rapid increase of intracellular free calcium concentration ([Ca$^{2+}$]) in fura-2/acetoxymethyl ester-loaded single glioma (T98G) cells. This increase was completely inhibited by removal of extracellular Ca$^{2+}$ with ethylene glycol bis(β-aminoethyl ether)-N,N,N,N-tetraacetic acid but not by an L-type calcium channel blocker (nicardipine), suggesting that PDGF may cause calcium influx through voltage-independent calcium channels in T98G cells. Trapidil and suramin blocked the PDGF-induced calcium response and inhibited the PDGF-initiated tyrosine phosphorylation of the PDGF receptor as detected by Western blot analysis using an antibody specific for phosphotyrosine. Trapidil and suramin also inhibited EGF-initiated calcium response in T98G cells, but only partially inhibited EGF-initiated tyrosine phosphorylation at the same concentrations. Our results suggest that trapidil and suramin inhibit PDGF- and EGF-initiated early biochemical events, and thus suppress growth factor-induced cell proliferation.

Key words: growth factor, calcium response, tyrosine phosphorylation, trapidil, suramin

Introduction

Growth factors may stimulate tumor growth through the autocrine or paracrine mechanisms. Platelet-derived growth factor (PDGF) and transforming growth factor-α (TGF-α) are produced by glioma cells, and their autocrine loops may be important for the proliferation of glioma cells. PDGF and epidermal growth factor (EGF) promote the proliferation of glioma cells in vitro, and exert their biological actions through activation of the receptor kinases, promoting many cellular alterations including the generation of signals that activate transcription and the tyrosine phosphorylation of specific intracellular substrates. Although the mitogen-initiated signals that trigger replication are not well understood, inhibitors that block one (or more) mitogen signals may inhibit the proliferation of tumor cells and may be clinically useful for the control of tumor growth.

Trapidil is a potent coronary vasodilator with an anti-aggregating effect, and inhibits PDGF-stimulated cell proliferation, possibly acting as a competitive antagonist at the PDGF receptor. Suramin, a polyanionic compound and an anti-parasitic agent used for treating African trypanosomiasis and onchocerciasis, inhibits the reverse transcriptase activity of ribonucleic acid tumor viruses or mammalian deoxyribonucleic acid polymerase. In addition, suramin may block the binding of growth factors to their receptors and subsequently inhibit proliferation of various types of...
cells. However, the mechanisms of these agents are not fully understood.

This study investigated the effects of trapidil and suramin on PDGF- and EGF-induced early biochemical events in a glioma cell line (T98G) established from human glioblastoma, using a single cell technique to detect mitogen-induced calcium response, and Western blot analysis to detect proteins containing phosphorylated tyrosine.

Materials and Methods

I. Cell culture

Human glioblastoma cells (T98G; American Type Culture Collection, Rockville, Md., U.S.A.) were grown in Dulbecco’s minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Flow Laboratories, McLean, Va., U.S.A.), 1% sodium pyruvate, 1% non-essential amino acids (Nissui Pharmaceutical Co., Tokyo), 100 IU/ml penicillin G, 100 μg/ml streptomycin, and 250 μg/ml amphotericin B under 5% CO2-95% air at 37°C.

II. Mitogen-induced calcium response

T98G cells in the exponential growth phase were subcultured on a glass coverslip in a 35-mm diameter culture dish at 2.5 × 10^3 cells/ml in culture medium under 5% CO2-95% air at 37°C. Cells on a coverslip were washed three times with DMEM and then serum-starved for 6 hours by incubating with culture medium lacking FBS. The cells were loaded with 2.5 μM fura-2/acetoxymethyl ester (Dojin Chemical Co., Tokyo) in serum-free DMEM containing 5% bovine serum albumin (fraction V; Miles Inc., Kankakee, Ill., U.S.A.) at 37°C for 30 minutes and washed three times with Hank’s balanced salt solution without phenol red (HBSS) (Nissui Pharmaceutical Co.). The cells attached to the coverslip were placed in a bath (volume 0.5 ml) on the stage of a fluorescence microscope (Optiphoto; Nikon, Tokyo) equipped with a photomultiplier tube (R649; Hamamatsu Electronics, Shizuoka), a photon counter (545A; NF, Hiroshima), and an appropriate combination of filters (Nihon Shinkuu Kougaku, Osaka). Alternating excitation was repeated with wavelengths of 340 (F340) and 380 nm (F380). Ratio values (F340/F380) were converted to cytosolic free calcium concentration ([Ca^{2+}]_i) by a calibration curve based on the fluorescence of acid-free fura-2 solution. As previously reported, the ratio of F340/F380 was linear with log[Ca^{2+}]_i over a wide range of 80-1000 nM. [Ca^{2+}]_i was calculated using a personal computer. HBSS at 37°C was continuously circulated at 1 ml/min. Cells on the coverslip were exposed to the various concentrations of recombinant human PDGF (R&D Systems Inc., Minneapolis, Minn., U.S.A.) or EGF (Higeta Shoyu Co., Chiba), FBS, or inhibitors at 37°C using circulation medium (HBSS) containing each agent as follows. All experiments were performed in triplicate.

PDGF-induced calcium response was examined by stimulating cells with 10 ng/ml PDGF. Extracellular calcium was then removed by circulating HBSS with 2.26 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N,N-tetraacetic acid (EGTA) (Dojin Chemical Co.), and cells were stimulated with 10 ng/ml PDGF. Cells were pretreated with 10 μM nicardipine (Yamanouchi Pharmaceutical Co., Tokyo) for 6 hours in serum-free DMEM in the CO2 incubator at 37°C, and then stimulated by 10 ng/ml PDGF. After stimulation by PDGF, the circulating medium was changed to HBSS containing 10% FBS as a positive control.

The effects of trapidil and suramin on PDGF-induced calcium response were examined by pretreatment of cells for 5 minutes with 10 or 100 μg/ml trapidil (Mochida Pharmaceutical Co., Tokyo), or 10 or 100 μg/ml suramin (Bayer AG, Wuppertal-Erlerfeld, Germany). The cells were then stimulated by 10 ng/ml PDGF or 10% FBS in the HBSS circulation medium containing the same concentration of trapidil or suramin.

The effects of trapidil and suramin on EGF-induced calcium response were studied by pretreatment of cells for 5 minutes with 10 or 100 μg/ml trapidil, or 10 or 100 μg/ml suramin, followed by stimulation by 100 ng/ml EGF or 10% FBS in HBSS circulation medium containing the same concentration of trapidil or suramin.

III. Protein tyrosine phosphorylation

T98G cells were cultured at 60-80% confluence in 35-mm-diameter culture dishes, then the medium was replaced with serum-free DMEM. After serum starvation for 16 hours in the absence or presence of trapidil or suramin, cells were treated with 10 ng/ml PDGF or 100 ng/ml EGF. The stimulation was terminated at various times by immediately aspirating the medium and adding a previously boiled electrophoresis sample buffer (100 μl/plate) consisting of 5 mM potassium phosphate (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10 mM dithiothreitol, 5% 2-mercaptoethanol, 0.04% bromophenol blue, and 2% glycerol. Cell lysates were collected with a rubber policeman, transferred to microcentrifuge tubes, then boiled for 2 minutes. The lysates were passed through a 22-gauge needle several times and then a 27-gauge needle 20 times. The extracted proteins (40
µg protein/lane) were separated by SDS-polyacrylamide gel electrophoresis in 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After blocking nonspecific binding sites with 4% milk casein (Block ACE™; Yukijirushi Co., Sapporo), the membrane was incubated with a monoclonal antibody specific for phosphotyrosine (clone Py54; Oncogene Sci., Uniondale, N.Y., U.S.A.). Bound antibodies were detected by enhanced chemiluminescence (Amer sham Japan, Tokyo).

Results

I. Mitogen-induced calcium response

The basal [Ca^{2+}]_i in fura-2/acetoxymethyl ester-loaded single cells was 193 ± 11 nM (mean ± SD, n = 17). Single cell analyses showed that a substantial proportion of cells (<23%) did not have a calcium response to PDGF, EGF, and FBS, and there were no obvious morphological features predicting which cells would be responsive. Therefore, FBS was always used as a positive control for calcium response, and data from cells unresponsive to FBS were omitted. The PDGF-induced increase of [Ca^{2+}]_i was 80.0 ± 90.4 nM (mean ± SD, n = 13). A typical response is shown in Fig. 1A. The PDGF-induced increase of [Ca^{2+}]_i was completely blocked when extracellular Ca^{2+} was removed with EGTA (Fig. 1B), indicating that PDGF evokes extracellular Ca^{2+} influx across the plasma membrane. However, the L-type calcium channel blocker, nicardipine, had no effect on the Ca^{2+} influx stimulated by PDGF (Fig. 1C), suggesting that the Ca^{2+} entry may not be through voltage-dependent calcium channels.

Trapidil at the concentrations of 10 and 100 µg/ml completely inhibited the PDGF-induced increase of [Ca^{2+}]_i (Fig. 2A, B), but did not inhibit the FBS

Fig. 1 PDGF-induced calcium response in single T98G cells. A: Typical PDGF-induced increase of [Ca^{2+}]_i. B: PDGF-induced increase of [Ca^{2+}]_i was completely blocked by removal of extracellular Ca^{2+} with EGTA. FBS was used as a positive control for calcium response. C: PDGF-induced Ca^{2+} response was not affected by nicardipine pretreatment. Results are representative of at least four separate experiments.

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response. Ten μg/ml suramin inhibited the PDGF-induced increase of [Ca^{2+}]i, but not in all experiments, as about 25% of the cells treated with 10 μg/ml suramin demonstrated a calcium response to PDGF (Fig. 2C). However, 100 μg/ml suramin completely blocked the PDGF-induced [Ca^{2+}]i increase. FBS was used as a positive control for calcium response. Results are representative of four separate experiments.

**II. Protein tyrosine phosphorylation**

PDGF stimulation of T98G cells caused prominent tyrosine phosphorylation of a protein with a molecular mass of 185 kd (Fig. 4, lanes 2 and 3), and probably represents the receptor for PDGF. Pretreatment of the cells with trapidil for 16 hours inhibited the PDGF-induced tyrosine phosphorylation of this protein (Fig. 4, lanes 4 and 5). Suramin also completely inhibited the PDGF-induced phosphorylation of the 185 kd protein (Fig. 4, lanes 6 and 7). In addition, a protein with molecular mass of 130 kd, which already contained phosphotyrosine before addition of PDGF and was not modified with PDGF (Fig. 4, lanes 1-3), showed tyrosine-dephosphorylation with both trapidil and suramin treatment (Fig. 4, lanes 4-7).

EGF induced rapid tyrosine phosphorylation of the 170 kd protein supposed to be the receptor for EGF and increased the modification of a 76 kd protein (Fig. 5, lanes 2–4), which was weakly tyrosine-phosphorylated before addition of EGF (lane 1).
Pretreatment of cells with trapidil only partially inhibited the EGF-induced tyrosine phosphorylation of 170 and 76 kd proteins (Fig. 5, lanes 5 and 6). This was also the case with suramin (Fig. 5, lanes 7 and 8). The phosphorylation of 170 and 76 kd proteins was only partially decreased with 100 and 200 μg/ml suramin, which completely inhibited the EGF-induced calcium response (Fig. 3). PDGF-induced calcium response and tyrosine phosphorylation were significantly inhibited in the presence of trapidil or suramin.

Fig. 3 Effects of trapidil and suramin on EGF-induced calcium response. A: Typical EGF-induced calcium response. Trapidil at 10 (B) and 100 μg/ml (C) and suramin at 10 (D) and 100 μg/ml (E) completely inhibited the EGF-induced increase of [Ca²⁺]i. FBS was used as a positive control for calcium response. Results are representative of four separate experiments.
suramin. EGF-induced tyrosine phosphorylation was not abolished, although EGF-induced calcium response was completely inhibited in the presence of trapidil or suramin.

**Discussion**

Our study found that PDGF induced increases in 
\([Ca^{2+}]_i\) of 80.0 ± 90.4 nM over the base level of 193 ± 11 nM in T98G glioma cells. The \([Ca^{2+}]_i\) levels of quiescent glioma cells were reported to be 100–200 nM.\(^{20}\) Our values were relatively high, but the values may vary depending on the instruments used.\(^{18}\) PDGF may cause a rapid increase in \([Ca^{2+}]_i\) by a dual mechanism: a transient increase in \([Ca^{2+}]_i\) results from an initial release of intracellular stores and a subsequent \(Ca^{2+}\) influx occurs across the plasma membrane.\(^{10,12,29}\) These studies were carried out using cells which express abundant PDGF receptors on the cell surface, and the responses may be dependent on the type of cells. We found that the PDGF-induced calcium response was abolished by removal of extracellular \(Ca^{2+}\) with EGTA, but not by treatment with nicardipine in T98G glioma cells. These results suggest that the influx of extracellular \(Ca^{2+}\) may be the main component of the PDGF-induced increase of \([Ca^{2+}]_i\), and that the influx is mediated by receptor-opened voltage-independent calcium channels and not by voltage-dependent channels.

Trapidil pretreatment of T98G cells completely inhibited the PDGF-induced calcium response, but not the FBS response. Pretreatment of T98G cells with trapidil also inhibited PDGF-initiated tyrosine phosphorylation of the 185 kd protein. In addition, PDGF stimulation of trapidil-treated cells caused dephosphorylation of the 130 kd protein, which previously contained phosphotyrosine. Characterization and identification of this protein may be the next important step to understand the actions of trapidil. 

Trapidil inhibited both the PDGF-initiated calcium response and tyrosine phosphorylation, supporting the concept that trapidil functions as a competitive inhibitor, binding preferentially to PDGF-receptor sites.\(^{23}\)

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**Fig. 4** Effects of trapidil and suramin on PDGF-induced protein tyrosine phosphorylation. Cell proteins were extracted before (lane 1), and 2 (lane 2) and 5 minutes (lane 3) after 10 ng/ml PDGF stimulation, and 5 minutes after stimulation from cells pretreated with 50 (lane 4) and 100 µg/ml trapidil (lane 5) or 100 (lane 6) and 200 µg/ml suramin (lane 7). Arrows indicate molecular masses calculated from the positions of molecular weight standards (MW STD). Similar results were obtained in three separate experiments.

**Fig. 5** Effects of trapidil and suramin on EGF-induced protein tyrosine phosphorylation. Cell proteins were extracted before (lane 1), and 1 (lane 2), 2 (lane 3), and 5 minutes (lane 4) after stimulation with 100 ng/ml EGF, and 5 minutes after stimulation from cells pretreated with 50 (lane 5) and 100,µg/ml trapidil (lane 6) or 100 (lane 7) and 200 µg/ml suramin (lane 8). Arrows indicate molecular masses of protein bands calculated from the position of molecular weight standards (MW STD). Results are representative of three separate experiments.
Trapidil is also reported to suppress the EGF-stimulated proliferation of meningioma cells *in vitro*. 3) Trapidil inhibited the EGF-induced calcium response but only partially decreased EGF-induced tyrosine phosphorylation of the 170 kd protein. These results suggest that trapidil may block not only the PDGF binding to its receptor but also inhibit the voltage-independent calcium channels that might be commonly activated by both PDGF and EGF.

Suramin inhibits the receptor binding of growth factors including PDGF, EGF, fibroblast growth factor, TGF-β, 2,5 and inactivates reverse transcriptase and deoxyribonucleic acid polymerase. 6,9,25 The effect of suramin on tyrosine phosphorylation varies depending on the type of cell studied, but inhibits 4,26 mitogen-induced tyrosine phosphorylation. We found suramin inhibited both PDGF-induced tyrosine phosphorylation of 185 kd protein and increase of [Ca^{2+}]_i in T98G cells, indicating inhibitory action at the receptor level. This agrees with the findings that suramin forms a complex with PDGF and dissociates PDGF from its receptor. 13 Suramin similarly inhibited the EGF-induced calcium response but did not abolish tyrosine phosphorylation.

In the present study, trapidil and suramin had similar inhibitory actions on PDGF- or EGF-initiated mitogen signals at the receptor level. Both agents blocked the increase of [Ca^{2+}]_i but did not inhibit tyrosine phosphorylation in response to EGF. This indicates that multiple pathways may be involved in the inhibitory actions of the two agents. Suramin-protein complex is taken up by cells, 4 and may inhibit signal transduction pathways including hydrolysis of phosphoinositides. 14

Our study confirms that trapidil and suramin may be clinically useful as an anti-cancer agents, and also suggests that these agents may be useful to study the biochemical signal events leading to proliferation of glioma cells *in vitro*.

**References**


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