Modulation of Prostaglandin Biosynthesis in Hypoxic Murine Mammary Adenocarcinoma Cells by Misonidazole

David R. Shalinsky, Dennis B. McNamara, and Krishna C. Agrawal

Department of Pharmacology, Tulane University School of Medicine, New Orleans, Louisiana 70112

ABSTRACT

Resistance of hypoxic cells to radiation and chemotherapy remains a major limitation to effective therapy of solid tumors. Misonidazole, a 2-nitroimidazole analogue, has been studied extensively as a radiosensitizer of hypoxic cells and has been shown to undergo bioreductive metabolism to exert preferential cytotoxicity against hypoxic cells. We have investigated the effects of misonidazole on the biosynthesis of prostaglandins (PGs) in murine mammary adenocarcinoma cell line (No. 4526) under aerobic and hypoxic conditions in attempts to exploit modulation of PG levels under hypoxia as a means of improving therapeutic approaches for the treatment of solid tumors. We report a time-dependent inhibition of PG biosynthesis by the suspended cells under hypoxia induced by flushing sealed vials with N₂ (1.5 liters/min). After 30 min of hypoxia, PG formation was inhibited by 50%. Indomethacin was able to further inhibit the PG formation in a concentration-dependent manner under hypoxia. Misonidazole, however, selectively increased the PGE₂ biosynthesis under hypoxia by 49% at 100 ìM. This increase was concentration dependent over the range of 25 to 100 ìM and was blocked by indomethacin (0.1 ìM). Misonidazole, the heterocyclic moiety in misonidazole without the nitro function, had no effect on PG biosynthesis at these concentrations. These data suggest that arachidonic acid metabolism is sensitive to the differential oxygen levels which exist within solid tumors and that PG levels may be modulated by electron-affinic agents in hypoxic tumor cells.

INTRODUCTION

Metabolism of AA has been associated with modulation of cell growth. Several reports have demonstrated growth-inhibitory activity for PGs of the E, A, D, and J series (1, 2). Administration of nonsteroidal antinflammatory agents such as indomethacin to mice bearing tumors has been shown to inhibit tumor growth (3) or to potentiate the effects of chemotherapeutic agents such as melphalan (4), further suggesting that modulation of PG levels may be beneficial in the treatment of cancer. The antiproliferative activity of these prostanooids seems to depend on the chemical transformation to the cyclopentone ring containing an α,β-unsaturated ketone (5, 6). It has been suggested that the mechanism of growth inhibition by these prostanooids does not seem to involve either the activation of adenylate cyclase or an increase in the levels of cyclic AMP (7). Recent reports have suggested that the uptake and intracellular accumulation of cyclopentone PGs in the nuclei may be responsible for their growth-inhibitory effects (2, 8). Inhibition of the synthesis of macromolecules such as DNA, RNA, and proteins by PGs has also been demonstrated (1, 9). However, inhibition of macromolecular synthesis by PGD₂ in a B16 melanoma cell line required higher concentrations than the concentrations needed to inhibit the replication of these cells in vitro (10). In contrast, elevated levels of PGs, notably PGE₂, have been associated with solid neoplasms in humans and animals, but it is not known whether these elevated levels reflect deregulated neoplastic biosynthesis or whether the excessive formation represents an endogenous mechanism for aiding tumor growth (11).

Since one of the major limitations to the effective treatment of solid tumors, e.g., of the colon, breast, or lung, is the presence of resistant hypoxic cells, it is of fundamental importance to investigate means of increasing the sensitivity of hypoxic cells to radiation or chemotherapy. These cells exist within the oxygen-deficient central core of the proliferating tumor (12) and have become hypoxic due to decreased oxygen diffusion from the peripheral blood supply. The hypoxic cells are generally not in the cell cycle and therefore are resistant to cell cycle-active chemotherapeutic agents (13) as well as to radiation, which requires the presence of oxygen for its full lethal effect. After radiation and/or chemotherapy of solid tumors, revascularization may induce the surviving hypoxic cells into cycling cells, thus enabling tumor regrowth.

Previous reports have suggested that synthesis of prostanoids in various tissues is stimulated by hypoxic conditions, such as low arterial Po₂ or ischemia (14). Recently, hypoxia has been shown to stimulate PGE₂ synthesis in renal mesangial cells in cultures maintained under chronic exposure to 2% oxygen (15, 16). However, PG biosynthesis in neoplastic cells under hypoxia has not been reported. Hence, we have investigated the influence of hypoxia on in vitro production of PGE₂ in murine mammary adenocarcinoma cells (line 4526) which have been shown to have a high level of activity of the cyclooxygenase pathway of arachidonic acid metabolism (17). Furthermore, it is not known whether the PG biosynthesis in neoplastic cells under hypoxia can be modulated by pharmacological agents. Since a class of electron-affinic compounds, the nitroimidazoles, such as misonidazole, has been shown to sensitize hypoxic cells to the effects of ionizing radiation and has been suggested to have oxygen-mimicking effects, it was anticipated that this class of compounds may modulate PG biosynthesis under hypoxia.

MATERIALS AND METHODS

Reagents. [1-¹⁴C]Arachidonic acid (40 to 60 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, MA). Prostaglandin standards and ibuprofen were obtained from The Upjohn Co. (Kalamazoo, MI). AA and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO). All ingredients needed for cell culture were purchased from Gibco (Grand Island, NY). Double-distilled water which had been filtered through a 0.22-μm filter was used in all studies.

Cell Culture. The murine mammary adenocarcinoma cell line (No. 4526) was derived from a lung metastasis of a BALB/c mouse bearing a spontaneously arising mammary adenocarcinoma tumor (18) and was obtained from Dr. Amy Fulton of the Michigan Cancer Foundation (Detroit, MI). The cells were grown in monolayer in 150-cm² tissue culture flasks (Costar, Cambridge, MA) in 10% heat-inactivated fetal bovine serum-supplemented Waymouth’s (MB 752/1) growth medium containing 250 units of penicillin/250 µg of streptomycin per ml, 0.025 mM L-glutamine and adjusted to pH 7.4 with a solution of NaHCO₃ (2.6 g/liter). The cells were kept in an air/CO₂ (95:5) incubator at 37°C and transferred (1/4) at confluency using standard tissue culture procedures. Cells from exponentially growing cultures were detached from...
the cells by using trypsin (2%)-EDTA (0.5%) and resuspended in growth medium to neutralize the action of trypsin. The cells were centrifuged (1100 x g for 5 min), and the pellet was washed in HBSS (pH 7.4), recentrifuged, and resuspended in HBSS to produce a cell suspension containing approximately 10^7 cells/ml. The cell number was obtained using a Coulter Counter. Cellular viability was assessed by using 0.4% trypan blue.

Assay of Arachidonic Acid Metabolism. The radiolabeled AA pool was prepared by mixing 0.9 nmol of [14C]AA with 1.1 nmol of AA in ethanol. The [14C]AA pool was then diluted with HBSS to provide 2 nmol of AA per 100-μl aliquot added to each incubation vial (120,000 dpm). Ethanol concentration did not exceed 0.1%.

One million cells suspended in 1.0 ml of HBSS in 10-ml glass vials were prewarmed to 37°C for 5 min and incubated for 20 min (or as noted) with 2.0 μM [14C]AA (2 nmol). In experiments performed under hypoxic conditions, vials containing cells were sealed using rubber stoppers. A flow of N₂ (1.5 liters/min) was maintained over the cell suspension through tubing connected to the stoppers via 22-gauge inlet and outlet needles. After 25 min when the cells have become hypoxic, the drug solution or HBSS was injected into the treated or control vials, respectively, for 5-min preincubation. Then, [14C]AA (in 100 μl) was injected through the stopper into each vial to initiate AA metabolism. Control vials containing cells for aerobic biosynthesis were also kept in the water bath for 30 min prior to incubation with [14C]AA. The reaction was stopped at appropriate time intervals, and the eicosanoids were extracted, separated, identified, and quantified as previously reported (19). A typical radiochromatographic scan was similar to those previously published (20).

Measurement of Dissolved Oxygen. DO level of HBSS in the vials was initially determined under experimental conditions without cells using a Rank Brothers oxygen electrode (Cambridge, England). The oxygen electrode was calibrated at the beginning of each experiment by adjusting to 100% DO level in HBSS (37°C) which was calculated to be 283 μM under basal conditions (21) and for 0% of oxygen after addition of dithionite, a compound which avidly reduces dissolved oxygen in solution. The dithionite was then washed out before starting the experiment. Nitrogen gas was passed through the air space over HBSS in the incubation vials via an inlet and outlet needle connection set up while monitoring the DO content. The electrode baseline was stable at the 100% DO level before monitoring the effect of the nitrogen gas using 1 x 10^6 cells suspended in the electrode chamber.

Statistical Analysis. The data are expressed as the group mean ± SEM of duplicate determinations from each “n”. Student's t test for grouped data was used. The 50% inhibitory concentration values were determined by probit analysis. In all cases, significance was at the level of P < 0.05.

RESULTS

Formation of PGs in Murine Mammary Adenocarcinoma Cells. The intactness of the plasma membrane as a marker for viability of the prepared cellular suspension was measured by exclusion of 0.4% trypan blue. Exclusion always exceeded 96% prior to and during incubation with arachidonic acid. The tumor cells were incubated with [14C]AA for varying time intervals (Fig. 1). A rapid rise in PGE₂ formation was observed for up to 20 min at the level of approximately 15% conversion of AA to PGE₂, after which there was no further significant increase in PGE₂ formation. The increases in the levels of PGF₂₅ and PGD₂ were relatively smaller. It is unlikely that the PG metabolites were reincorporated into the cells since the outer cell membrane has been generally reported to be impermeable to their inward diffusion (22). However, a relatively smaller decrease in the formation of all three prostanooids at 60 min may represent catabolism by 15-hydroxyprostaglandin dehydrogenase. Formation of 6-keto-PGF₁α, or thromboxane B₂ was not detected in extracts of the cells. Subsequent studies used the 20-min reaction period by which time the formation of PGE₂ had reached a plateau.

Effect of Inhibitors of Cyclooxygenase. The cells were preincubated for 30 min with either indomethacin (10⁻⁹ to 10⁻⁶ M) or ibuprofen (10⁻⁹ to 10⁻⁷ M) prior to metabolism of arachidonic acid. Both cyclooxygenase inhibitors decreased the formation of PGE₂, PGD₂, and PGF₂₅ in a concentration-dependent manner as shown for indomethacin in Fig. 2. The data for ibuprofen are not shown. The 50% inhibitory concentration value for PGE₂ inhibition was 6.3 × 10⁻⁸ M and 9.6 × 10⁻⁸ M for indomethacin and ibuprofen, respectively.

Effects of Hypoxia on PG Biosynthesis. The dissolved oxygen level in the vials was determined under experimental hypoxic and aerobic conditions (Fig. 3). The baseline for 0% dissolved oxygen was determined by addition of dithionite, which avidly reduces dissolved oxygen and immediately decreased its level in the chamber to 0%. After the dithionite was washed out and a 10-min period to monitor baseline stability, nitrogen flow (1.5

---

**Fig. 1.** Formation of prostaglandins in mammary adenocarcinoma cells as a function of time. Suspended cells (1 x 10⁶) in HBSS were incubated with 2.0 μM [14C]AA for varying time intervals. Columns, mean; bars, SEM (n = 20).

**Fig. 2.** Effect of indomethacin on prostaglandin formation in mammary adenocarcinoma cells. Suspended cells (1 x 10⁶) were preincubated with drug for 30 min prior to incubation with 2.0 μM [14C]AA for 20 min. Points, mean; bars, SEM (n = 6).

**Fig. 3.** Measurement of DO levels under experimental conditions. At Arrow 1, dithionite was added into the electrode chamber to deplete the DO. At Arrow 2, an N₂ flush (1.5 liters/min) was initiated through the sealed vials, and the DO had dropped by 87% after 30 min (Arrow 3). At Arrow 4, the DO baseline was monitored under aerobic conditions. A representative chromatogram is shown (n = 3).
were incubated for 30 min with indomethacin prior to incubation with [I4C]AA for 20 min. Columns, mean; bars, SEM (n = 6). *, P < 0.05 with respect to hypoxic conditions. Hypoxia was induced by 30-min flush with N2 (1.5 liter/min). Cells were exposed to hypoxic controls shown under zero concentration.

Carcinoma cells. Cells (1 x 10⁶) were exposed to increasing periods of hypoxia (Fig. 7).

Sis was inhibited by approximately 40% of hypoxic control at 25 uM. However, at 500 uM and higher concentrations of misonidazole, PGE₂ formation was attenuated. Hypoxic PGF₂α production was also significantly increased in the presence of 25 uM misonidazole, but was not increased consistently. Under hypoxic conditions, indomethacin (0.1 uM) completely blocked the enhanced PG biosynthesis produced by misonidazole (100 uM). At this concentration of indomethacin, PG biosynthesis was inhibited by approximately 40% of hypoxic control (Fig. 7).

Effect of Misonidazole on PGE₂ Biosynthesis. The effect of misonidazole on PG biosynthesis under hypoxic conditions is shown in Fig. 6. Under aerobic conditions, misonidazole did not alter PG biosynthesis (data not shown), whereas under hypoxia, misonidazole increased the formation of PGE₂ by 49% in a concentration-dependent manner over the range of 25 to 100 uM. However, at 500 uM and higher concentrations of misonidazole, PGE₂ formation was attenuated. Hypoxic PGF₂α production was also significantly increased in the presence of 25 uM misonidazole, but was not increased consistently. Under hypoxic conditions, indomethacin (0.1 uM) completely blocked the enhanced PG biosynthesis produced by misonidazole (100 uM). At this concentration of indomethacin, PG biosynthesis was inhibited by approximately 40% of hypoxic control (Fig. 7).

DISCUSSION

The 4526 murine mammary adenocarcinoma cell line was shown to biosynthesize primarily PGE₂, and this formation was inhibited in a concentration-dependent manner by cyclooxygenase inhibitors such as indomethacin and ibuprofen, suggesting that PGE₂ formation in this model system was enzymatic. However, as PGE₂, PGF₂α, and PGD₂ can be formed spontaneously by hydrolysis of their precursor, PGH₂, demonstration of the presence of the isomerase that forms PGE₂ is required in order to determine whether or not the PGE₂ formation in this model was enzymatic. A time-dependent inhibition of PGE₂ formation was observed under hypoxia and was reversed selectively by misonidazole (25 to 100 uM) in a concentration-dependent manner. The enhanced PGE₂ biosynthesis by misonidazole under hypoxia was completely blocked by indomethacin, suggesting that misonidazole does not act independently of cyclooxygenase. The conditions used for the induction of hypoxia in these experiments were similar to those used previously.

At the maximum concentration studied, misonidazole neither affected cellular viability as determined by the intactness of plasma membrane to trypan blue exclusion nor was the growth rate of these cells altered (data not shown). Imidazole at equimolar concentrations did not change prostaglandin formation under aerobic or hypoxic conditions. Imidazole also did not affect cellular viability as measured by trypan blue staining (data not shown).
for hypoxic cytotoxicity and radiosensitization studies (23).

Incubation of the tumor cells with exogenously added AA resulted in the formation of PGE₂, PGF₂α, and PGD₂ similar to other studies (17). No evidence of prostacyclin or thromboxane formation was observed as indicated by the absence of formation of the stable hydrolytic products 6-keto-PGF₁α or thromboxane B₂, respectively. Additional studies using sonicated cells to metabolize the endoperoxide substrate, PGH₂, have demonstrated the presence of the reduced glutathione-dependent PGE₂ isomerase in these cells (24). These studies failed to demonstrate the presence of reduced glutathione-dependent or -independent PGH₂ to PGD₂ isomerase or the enzymatic conversion of PGH₂ to PGF₂α; therefore, it seems likely that the formation of PGF₂α and PGD₂ in this model is secondary to hydrolysis of PGH₂.

After 30-min flushing with nitrogen through the sealed vials, the DO level decreased to 13% of control (37 μM) and continued to drop over time. The Kᵣ of oxygen for cyclooxygenase has been reported to be 5 μM (25), suggesting the observed inhibition of PGE₂ biosynthesis under hypoxia was due to the decreasing DO level over the incubation period. Prostaglandin formation was inhibited by 50% under these conditions. After 120 min of N₂ flushing when the oxygen content decreased to the 0% level similar to that produced by dithionite, the PGE₂ biosynthesis still remained higher than expected at approximately 20% of control values, suggesting that amounts of air sufficient to support cyclooxygenase activity might have been introduced with injection of substrate into the sealed vials. Since the controls for hypoxic vials received the same protocol as the drug-treated groups, any extra oxygen inserted during the injection of drug into the vial would have similar effects for each group.

Misonidazole selectively augmented the hypoxic PGE₂ formation without affecting aerobic PGE₂ biosynthesis up to a concentration of 100 μM. In contrast, the concentrations of misonidazole required for radiosensitization of hypoxic cells are in the range of 0.25 to 1 mM or higher. Moreover, the hypoxic cytotoxicity due to misonidazole is observed only at concentrations of 1 mM or higher and requires a longer incubation time of more than 4 h (23). It may be postulated that reduction of the nitro group was coupled to the oxidation of arachidonic acid, since the nitro function has been observed to undergo rapid reduction under these conditions (26). Alternatively, misonidazole may have acted as an oxygen-mimicking agent as a radical anion (27) to enhance the conversion of AA to PGH₂ with subsequent metabolism to PGE₂. When the cells were incubated under hypoxia, with indomethacin (0.1 μM) in the presence of misonidazole (100 μM), the resulting inhibition of enhanced PGE₂ biosynthesis indicated that the increase in synthesis under hypoxia was due to the cyclooxygenase activity. These data also suggest that misonidazole is not able to protect the cyclooxygenase activity from the inhibitory effect of indomethacin. The parent molecule, imidazole, which lacks the nitro function, did not elevate hypoxic PGE₂ formation, indicating the necessity of the electron-affinic group. These data demonstrate that AA metabolism is sensitive to the differential oxygen levels which exist within solid tumors and that PG levels may be modulated by electron-affinic agents in hypoxic tumor cells.

ACKNOWLEDGMENTS

Great appreciation is expressed to Dr. Amy Fulton of the Michigan Cancer Foundation for kindly supplying the tumor cell line and to Suzanne M. Knoop and Barbara J. Rider for assistance in the analysis of these studies.

REFERENCES

Modulation of Prostaglandin Biosynthesis in Hypoxic Murine Mammary Adenocarcinoma Cells by Misonidazole

David R. Shalinsky, Dennis B. McNamara and Krishna C. Agrawal