Volume 316, number 1, 99–102 FEBS 12025 January 1993

Thromboxane A_2 analogue U 46619 enhances tumour cell proliferation in HeLa cells via specific receptors which are apparently distinct from TXA_2 receptors on human platelets

Santosh Nigam, Saeed Eskafi, Almut Roscher and Hans Weitzel

Eicosanoid Research, Department of Gynecology, Klinikum Steglitz, Free University Berlin, D-1000 Berlin 45, Germany

Received 10 November 1992; revised version received 9 December 1992

In this study, we have demonstrated for the first time by using U 46619, a stable analogue of thromboxane A_2 (TXA_2), that TXA_2 exerts a cell proliferative effect on HeLa cells which is mediated by specific TXA_2 receptors, inasmuch as the cell proliferation could be dose-dependently suppressed by TXA_2 receptor antagonist BM 13177. The investigation of the phospholipase C pathway by U 46619 and prostaglandin H_2 (PGH_2) in the presence and absence of BM 13177 in cells with or without pertussis toxin pretreatment, as well as radioligand receptor binding studies, revealed that, in contrast to TXA_2 receptors on human platelets, where TXA_2 and PGH_2 share the same receptor binding sites, HeLa cells possess distinct receptors for TXA_2 and PGH_2.

HeLa cell; Thromboxane A_2; U 46619; BM 13177; TXA_2 receptor; Cell proliferation

1. INTRODUCTION

There exists plenty of evidence that growing tumours synthesize eicosanoids ([1], and references therein) and that endogenously produced eicosanoids participate in the modulation of tumour growth [2–6] and metastasis [4,7]. In addition, it has been reported that TXA_2 is preferentially formed during tumour growth in animal and humans [8]. The recent finding that TXA_2 synthetase activity is increased in growing tumours of human breast [9] provided more support for the specific role of TXA_2 in tumour growth.

In the present paper, we report for the first time that the stable analogue of TXA_2, U 46619, accelerates cell proliferation in HeLa cell cultures through a specific TXA_2 receptor. Furthermore, this TXA_2 receptor is apparently distinct from the TXA_2 receptor on human platelets.

2. MATERIALS AND METHODS

2.1. *Materials*

Eicosanoids and U 46619 were purchased from Cayman Chemicals, Ann Arbor, USA. Indomethacin, FURA-2/AM were supplied by Sigma, Germany, and pertussis toxin by Calbiochem, Germany. Sodium meclofenamate was a gift from Warner Lambert, Ann Arbor, USA, and the TXA_2 receptor antagonist, BM 13177, a gift from Dr. Stegmeier from Boehringer, Mannheim, Germany. SQ 29548 was supplied by Cayman Chemicals, Ann Arbor, USA. Radiolabeled compounds were purchased from Amersham, Germany.

Correspondence address: S. Nigam, Eicosanoid Research, Department of Gynecology, Universitätsklinikum Steglitz, Free University Berlin, D-1000 Berlin 45, Germany. Fax: (49) (30) 815 5510.

This paper is dedicated to the Late Professor Trevor F. Slater, Department of Biochemistry, Brunel University, Uxbridge, Middlesex, UK.

2.2. *Cultivation of HeLa cells*

HeLa cells were cultured in Minimum Eagle's Medium (MEM), supplemented with 10% fetal calf serum (FCS), 1% L-Gln (200 mM), 0.5% penicillin (10 000 U/ml) and 0.5% streptomycin (10 000 μg/ml) at 37°C in an incubator (5% CO_2). Confluency was obtained in 48 h.

Cells were obtained as single-cell suspensions by rinsing the monolayers with phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+} before trypsinizing with trypsin/EDTA (0.05/0.02%, w/w) for 5 min at 37°C. Cells were washed twice with PBS, resuspended in MEM and supplemented with FCS, L-Gln and penicillin/streptomycin as above. The viability of cells was ≥ 96%.

2.3. *Cell proliferation assay*

The influence of different effectors on tumour cell proliferation was assayed by means of [^{3}H]thymidine (sp. act. 20.1 Ci/mmol) uptake for 6 h at 37°C in an incubator.

2.4. *Radioligand receptor binding assay*

Cell suspensions were obtained as described above and adjusted to a density of 2×10^6 cells/ml medium. Aliquots (50 μl) of the labeled TXA_2 analogue, [^{3}H]U 46619 (sp. act. 12.1 Ci/mmol), and unlabeled compounds, e.g. U 46619, TXB_2, PGH_2 (prostaglandin H_2), 11-dehydro-TXB_2, and PGE_2 were added to 1 ml of cell suspension (pH 7.4) to obtain concentrations of various substances in the suspension between 1 nM and 10 μM. Incubation was carried out for 1 h at 37°C in an incubator. The reaction vials were then centrifuged at $100 \times g$ for 5 min, the pellet was washed twice and gently resuspended in 500 μl PBS. The radioactivity was counted in a scintillation counter (Beckman, USA).

Specific binding was determined as the difference between the amount of [^{3}H] U 46619 bound to the cells in the absence and presence of 10 μM U 46619. All experiments were performed in triplicate.

2.5. *Measurement of intracellular Ca^{2+} $[Ca^{2+}]_i$*

Intracellular Ca^{2+} in HeLa cells was measured as described [10].

Briefly, HeLa cells (5×10^6 cells/ml) were incubated with 5 μM FURA-2/AM for 45 min at room temperature. After washing, cells were resuspended in PBS. The cells were then challenged with various stimuli as indicated in section 3. The fluorescence was measured in a Hitachi F-4000 spectrophotometer using excitation and emission wavelengths at 340 nm and 505 nm, respectively. Concentrations of $[Ca^{2+}]_i$ were calculated as described elsewhere [11].

3. RESULTS AND DISCUSSION

Upon treatment with the TXA_2 analogue, U 46619, freshly cultured HeLa cells demonstrated an enhanced cell proliferation dose-dependently as measured by [^{3}H]thymidine uptake (Fig. 1A). At a concentration of 1 μM almost 145% of cell proliferation was achieved. Moreover, the proliferative effect of U 46619 was not imitated by other TXA_2 degradation products and prostanoids, particularly PGH_2, which shares several properties of TXA_2 related to platelet functions [12].

Considering the recent finding that the growing breast tumours show increased activity of TXA_2 synthetase [9], one would expect the suppression of tumour growth by successful application of TXA_2 inhibitors in vitro and in vivo. Our results with inhibitors of cyclooxygenase enzyme, shown in Fig. 1B, do not support the previous reports [7]. Whereas indomethacin and sodium meclofenamate were unable to inhibit cell proliferation, even at a concentration of 20 μM, the proliferative effect of U 46619 could be suppressed dose-dependently by the TXA_2 receptor antagonists, BM 13177 (IC_{50} = 0.5 μM) and SQ 29548 (IC_{50} = 0.92 μM). This gives a clear cut evidence that U 46619 exerts a receptor-mediated cell proliferative effect on HeLa cells. Furthermore, it explains why treatment of tumour cells with cyclooxygenase inhibitors did not prevent the cell proliferation, since these inhibitors were incapable of achieving a complete suppression of cellular TXA_2 synthesis [13]. Consequently, a residual TXA_2 synthesis is sufficient for receptor-mediated tumour cell proliferation.

U 46619 has also been shown to stimulate phospholipase C (PLC) via receptor G-protein coupling in several cell types [14,15]. Fig. 2A shows the effect of U 46619 on the release of $[Ca^{2+}]_i$ in HeLa cells. A rapid and transient release of $[Ca^{2+}]_i$ was observed when cells were challenged with U 46619. The release of $[Ca^{2+}]_i$ was dose dependent and could be suppressed by pretreatment of cells with 10 μM BM 13177 (Fig. 2A, inset and lower panel). Suppression of $[Ca^{2+}]_i$ was also obtained when HeLa cells were pretreated with pertussis toxin for 90 min at 37°C (Fig. 2B), suggesting the involvement of a G-protein. This indicates that the TXA_2 receptors on HeLa cells are apparently distinct from TXA_2 receptors on human platelets, inasmuch as the platelet TXA_2 receptors are coupled to a pertussis toxin-insensitive G-protein [16,17].

Surprisingly, HeLa cells which were already challenged with PGH_2, were still capable of releasing $[Ca^{2+}]_i$ by treatment with U 46619 (Fig. 3A). Again, whereas

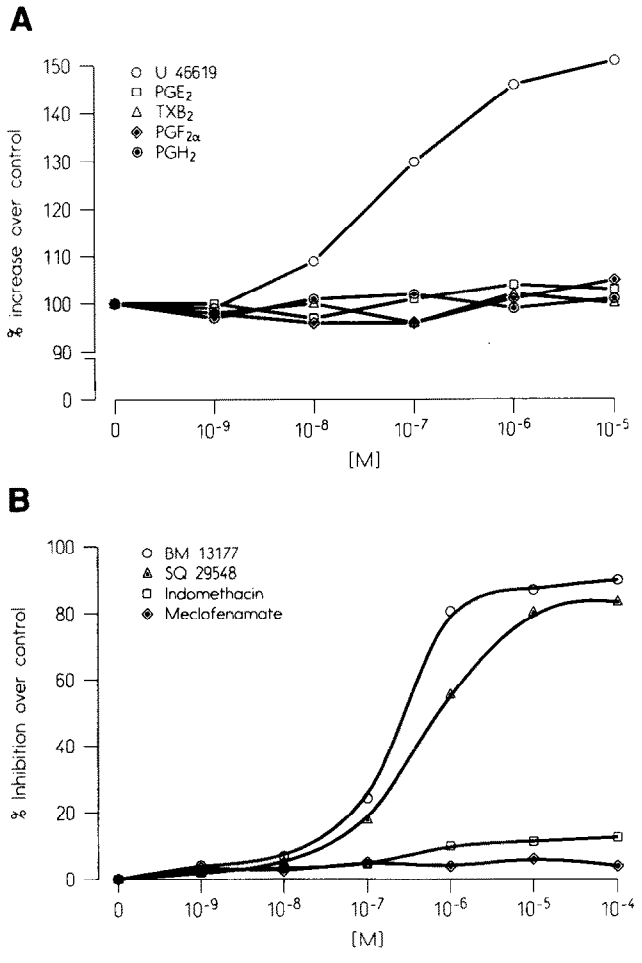

Fig. 1. (A) Effect of U 46619 and other prostanoids on the [^{3}H]thymidine incorporation by HeLa cells. Values are expressed as a mean of 7 separate cultures. (B) Effect of cyclooxygenase inhibitors and TXA_2 receptor antagonists on the [^{3}H]thymidine incorporation by HeLa cells. Values are expressed as a mean of 7 separate cultures.

pretreatment of HeLa cells with 1 μM BM 13177 significantly suppressed the U 46619-induced $[Ca^{2+}]_i$ release, no inhibitory effect of BM 13177 was observed on the PGH_2-induced $[Ca^{2+}]_i$ release (Fig. 3B), suggesting distinct receptor binding sites for TXA_2 and PGH_2.

Fig. 4 shows the radioligand binding experiments with [^{3}H]U 46619. Whereas increasing concentrations of unlabeled U 46619 blocked the specific binding of [^{3}H]U 46619, none of the degradation products of TXA_2 or other prostanoids effectively altered the binding of [^{3}H]U 46619. PGH_2, which shares chemical identity with TXA_2, did partially block [^{3}H]U 46619 binding, albeit at very high concentrations (not shown).

We, therefore, conclude that TXA_2 exerts a cell proliferative effect on HeLa cells which is mediated via specific TXA_2 receptors and can be totally blocked by TXA_2 receptor antagonists. In addition, we found that, in contrast to TXA_2 receptors on platelets, the receptors on HeLa cells are not shared by PGH_2 and are coupled

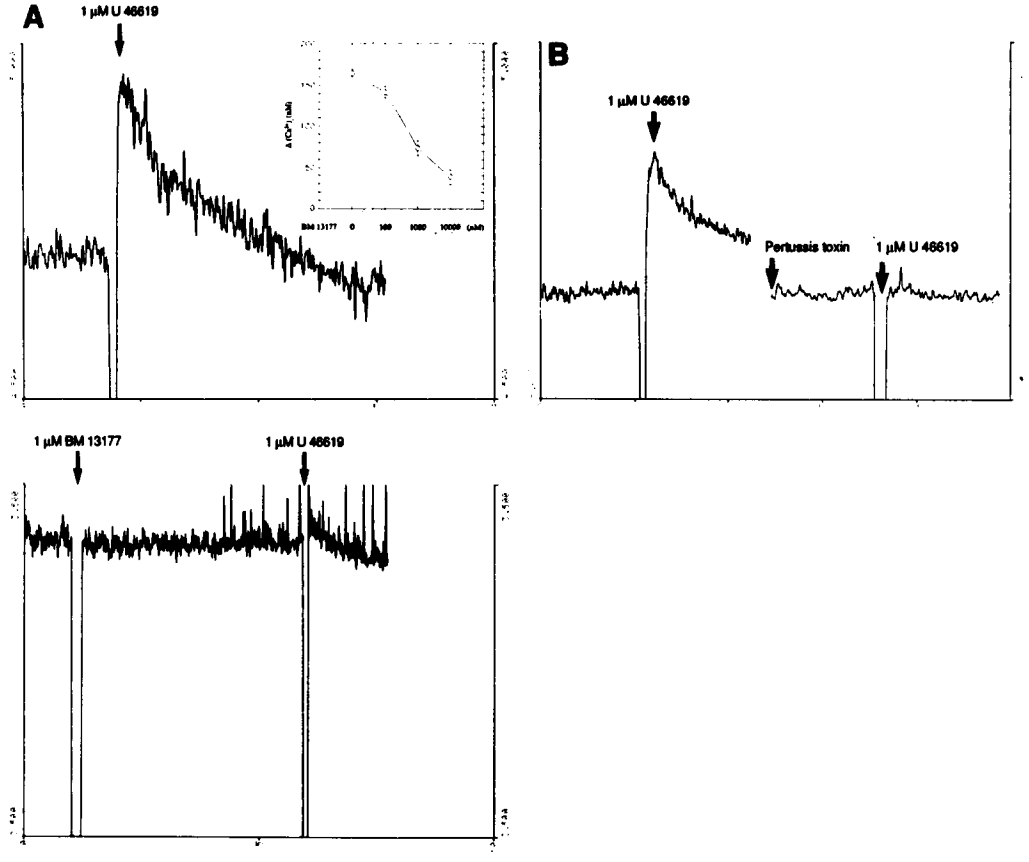

Fig. 2. (A) Effect of U 46619 on the release of $[Ca^{2+}]_i$ in HeLa cells. The tracing represents 1 of 5 separate experiments with identical results. (Inset) Dose-dependent inhibition of $[Ca^{2+}]_i$ by BM 13177. Values are expressed as mean ± S.E.M. of 3 separate experiments. (Lower panel) Inhibition of $[Ca^{2+}]_i$ by 1 μM BM 13177. The tracing represents 1 of 5 separate experiments with identical results. (B) Effect of pertussis toxin on the U 46619-induced release of $[Ca^{2+}]_i$. The tracing represents 1 of 3 separate experiments with identical results.

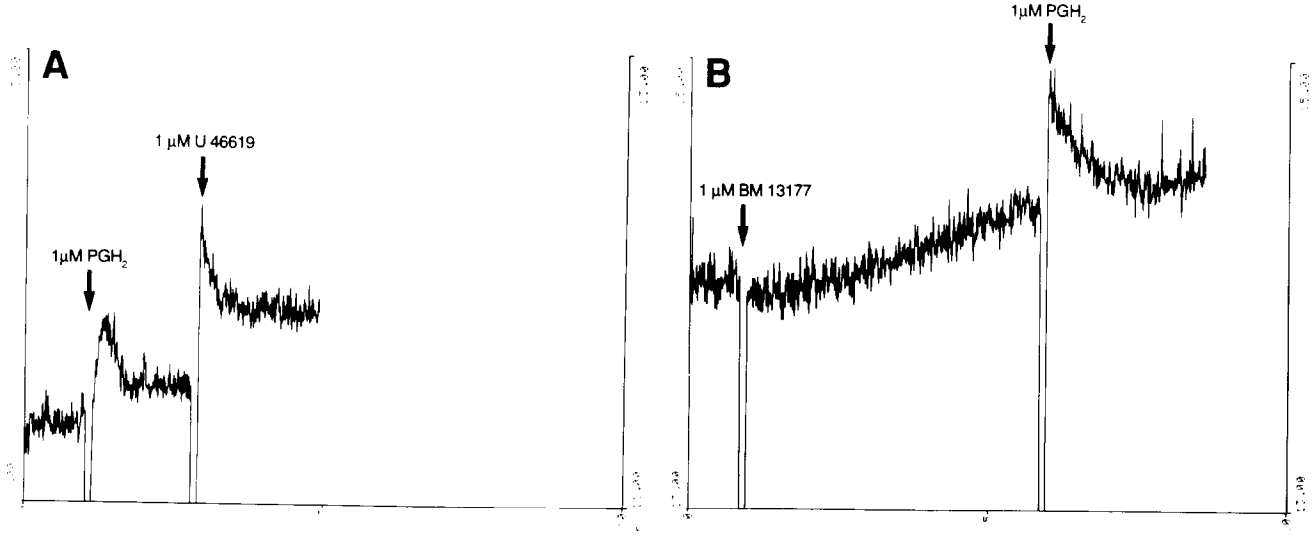

Fig. 3. Evidence for distinct binding sites of U 46619 and PGH_2 in HeLa cells for the release of $[Ca^{2+}]_i$ (A and B). The tracing represents 1 of 3 separate experiments with identical results.

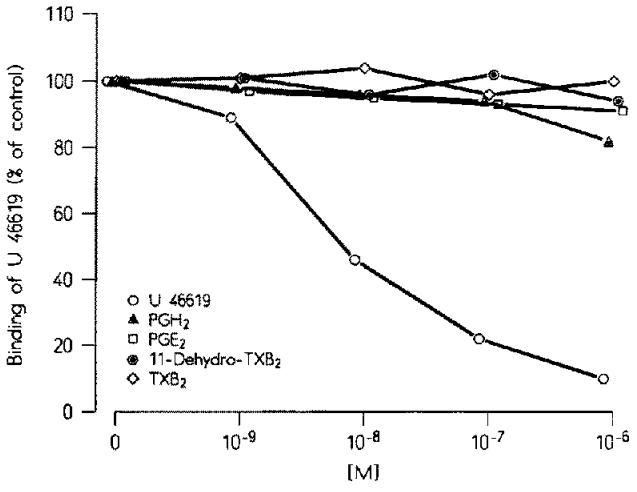

Fig. 4. Specific binding of [^{3}H]U 46619 and other prostanoids to TXA_2 receptor binding sites. Results are expressed as a mean of 4 separate experiments with triplicate determinations.

to PLC via a pertussis toxin-sensitive G-protein. As a consequence of our data, we suggest that an additional classification of tumours between TXA_2 receptor-positive and TXA_2 receptor-negative ones in analogy to estrogen/progesterone receptor-positive and estrogen/progesterone-negative tumours should be introduced. This may have far reaching implications for a therapeutic approach for prevention of tumour growth, inasmuch as a number of TXA_2 receptor antagonists have been developed in the past few years and many of them are already available on the market.

Acknowledgements: This study was generously supported by the Association for International Cancer Research, UK (Grant Ni/81025). The authors wish to thank Prof. G. Wurm (Free University, Berlin) for helpful discussions, and Andreas Zakrzewicz, Emily Willkomm and Martina Giermann for technical assistance.

REFERENCES

[1] Nigam, S., Zakrzewicz, A., Eskafi, S. and Roscher, A. (1992) Cancer Metastasis Rev. 11, 411–420.
[2] Jimenez de Asua, L., Richmond, K.M. and Otto, A.M. (1981) Proc. Natl. Acad. Sci. USA 78, 1004–1008.
[3] Honn, K.V., Bockman, R.S. and Marnett, L.J. (1981) Prostaglandins 21, 833–863.
[4] Honn, K.V. and Meyer, J. (1981) Biochem. Biophys. Res. Commun. 102, 1122–1129.
[5] Fletcher R.J. (1989) in: Prostaglandins in Clinical Practice (Watkins, W.D., Petersen, M.B., Fletcher, R.J. eds.) Raven Press, New York, p. 59.
[6] Nigam, S. and Zakrzewicz, A. (1990) Adv. Prostagl. Thrombox. Leukotr. Res. 21, 925–928.
[7] Honn, K.V., Busse, W.D. and Sloane, B.F. (1983) Biochem. Pharmacol. 32, 1–11.
[8] Chiabrando, C., Broggini, M., Castelli, M.G., Cozzi, E., Castagnoli, M.N., Donell, M.G., Garattini, S., Giavazzi, R. and Fanelli, R. (1987) Cancer Res. 47, 988–991.
[9] Nüsing, R., Lesch, R. and Ullrich, V. (1990) Eicosanoids 3, 53–58.
[10] Nigam, S., Müller, S. and Walzog, B. (1992) Biochim. Biophys. Acta 1135, 301–308.
[11] Scanlon, M., Williams, D.A. and Fay, F.S. (1987) J. Biol. Chem. 262, 6308–6312.
[12] Halushka, P.V., Mais, D.E., Mayeux, P.R. and Morinelli, T.A. (1989) Annu. Rev. Pharmacol. Toxicol. 29, 213–239.
[13] Nigam, S. and Averdunk, R. (1988) in: Eicosanoids, Lipid Peroxidation and Cancer (Nigam, S., McBrien, D.C.H., Slater, T.F. eds.), Springer Verlag, Heidelberg, p. 43.
[14] Nakahata, N., Matsuoka, I., Ono, T. and Nakanishi, H. (1989) Eur. J. Pharmacol. 162, 407–417.
[15] Mene, P., Dubyak, G.R., Abboud, H.E., Scarpa, A. and Dunn, M.J. (1988) Am. J. Physiol. 255, F1059–F1069.
[16] Houslay, M.D., Bojanic, D. and Wilson, A. (1986) Biochem. J. 234, 737–742.
[17] Brass, L.F., Schaller, C.C. and Belmonte, E.J. (1987) J. Clin. Invest. 79, 1269–1278.