Volume 290, number 1,2, 239–242 FEBS 10196 September 1991

ADONIS 001457939100909S

Tunicamycin inhibits the initiation of DNA synthesis stimulated by prostaglandin $F_{2\alpha}$ in Swiss mouse 3T3 cells

Alvaro G. Estevez, Gabriela Calamante, Mercedes Goin and Luis Jimenez de Asua

Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI), Obligado 2490, 1428 Buenos Aires, Argentina

Received 3 June 1991; revised version received 31 July 1991

Tunicamycin, an inhibitor of the asparagine-linked protein *N*-glycosylation, blocks the initiation of DNA synthesis in Swiss 3T3 cells stimulated by prostaglandin $F_{2\alpha}$ alone or with insulin. This effect is exerted only when tunicamycin is added from 0 to 8 h after stimulation and it decreases the rate of entry into S phase. Blocking of labeled sugar incorporation to proteins occurs regardless of the time of $PGF_{2\alpha}$ stimulation. In contrast tunicamicin does not inhibit protein synthesis. These results suggest that *N*-glycoprotein synthesis early during the prereplicative phase is an important event controlling the mitogenic action of $PGF_{2\alpha}$.

Swiss 3T3 cell; Prostaglandin $F_{2\alpha}$; Tunicamycin; DNA synthesis; Lag phase

1. INTRODUCTION

Mammalian cell proliferation involves the expression of a reproducible program of signals and intracellular events which precede the onset of DNA replication [1–3]. Postranslational protein modifications have been shown to be involved in the regulation of cell division [4]. The *N*-glycosylation of newly synthesized proteins plays an important role in controlling embryonic development, cell differentiation and proliferation [5–14].

N-Glycosylation involves several metabolic steps leading to the formation of the dolichol core oligosaccharide and its transference to the nascent polypeptide chains [15]. Tunicamycin (TM) inhibits the first step of the *N*-glycosylation process [16] and thereby alters many cellular processes [5–11].

Cultured Swiss mouse 3T3 cells have provided a useful model system to study the mechanisms that regulate proliferation [17]. These cells can be arrested in the G_0/G_1 phase of the cell cycle upon serum deprivation or when allowed to become confluent [2,3]. Addition to such cultures of serum or a variety of growth factors, including prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), stimulates the initiation of DNA synthesis after a constant lag phase of 14–15 h [2,3]. The rate of entry into S phase follows apparent first-order kinetics and can be quantified by a rate constant *K* [18,19].

Here we show that in confluent resting Swiss mouse 3T3 cells stimulated by $PGF_{2\alpha}$ without or with insulin, TM inhibits the initiation of DNA synthesis, only if added within the first 8 h after mitogenic induction. Nevertheless, TM does reduce the incorporation of [^{14}C]glucosamine and [^{3}H]mannose into total cellular protein at any time after stimulation. These results suggest that *N*-glycoprotein synthesis early during the lag phase plays an important role in the mitogenic response of $PGF_{2\alpha}$.

Abbreviations: $PGF_{2\alpha}$, prostaglandin F2α; TM, tunicamycin.

Correspondence address: L. Jimenez de Asua, INGEBI, Obligado 2490, 1428 Buenos Aires, Argentina.

2. MATERIALS AND METHODS

2.1. *Cell culture, initiation of DNA synthesis assay and determination of rate constant for entry into S phase*

Cell culture, conditions for the assay of DNA synthesis, labeling with [methyl-^{3}H]thymidine and autoradiography were performed as previously described [19]. The value of the rate constant *K* for entry into S phase was calculated as before [19].

2.2. *Glucosamine, mannose and leucine incorporation*

Cells were plated as for the assay of DNA synthesis [19]. For sugar labeling, the culture medium was removed and the cells were washed twice with 2.0 ml of serum-free medium minus glucose pre-warmed to 37°C. The cells then received 2.0 ml of the same medium containing the different stimuli and were labeled with 25 μM [^{14}C]glucosamine (1 μCi/ml) and 50 μM [^{3}H] mannose (2.5 μCi/ml) as indicated. For protein synthesis, stimulated cultures were exposed to [^{3}H]leucine (2.5 μCi/ml). Thereafter, cells were processed and radioactivity was counted as described before [13].

2.3. *Materials*

$PGF_{2\alpha}$ was the generous gift of Dr. John Pike, Upjohn Co. All remaining chemicals were purchased from Sigma. D-[2-^{3}H]mannose (10-20 Ci/mmol), D-[N-^{14}C]glucosamine hydrochloride (250–350 mCi/mmol), L-[4,5-^{3}H]leucine (130 Ci/mmol) and [methyl-^{3}H]thymidine (18 Ci/mmol) were from the Radiochemical Center Amersham.

3. RESULTS

3.1. *Tunicamycin inhibition of DNA synthesis*

The effect of TM on the initiation of DNA synthesis

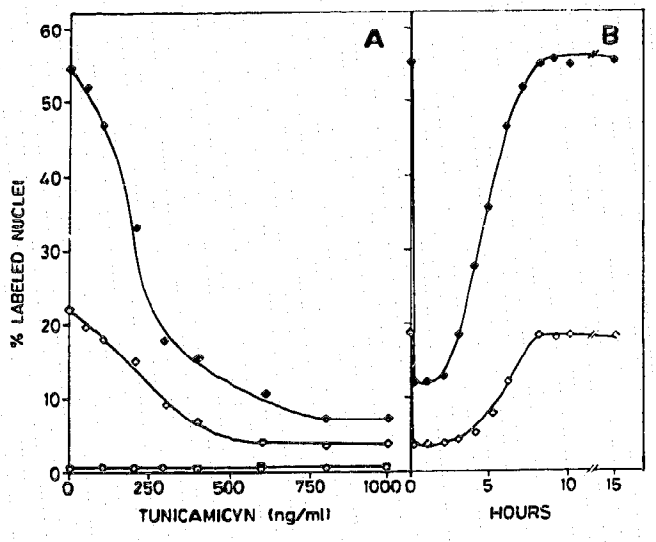

Fig. 1. (A) Effect of TM on the stimulation of DNA synthesis by $PGF_{2\alpha}$ (300 ng/ml) without or with insulin (60 ng/ml). (◇), $PGF_{2\alpha}$ plus TM; (◆), $PGF_{2\alpha}$ plus insulin and TM; (○), TM; (□), TM plus insulin. (B) Effect of TM (250 ng/ml) added at different times after $PGF_{2\alpha}$ (300 ng/ml) without or with insulin (60 ng/ml) on the stimulation of DNA synthesis. (◇), $PGF_{2\alpha}$ with TM; (◆), $PGF_{2\alpha}$ with insulin plus TM. Cultures were labeled for autoradiography from 0 to 28 h after additions as indicated in Materials and Methods.

is shown in Fig. 1A. $PGF_{2\alpha}$ (300 ng/ml) added to confluent resting Swiss 3T3 cells stimulates the initiation of DNA synthesis, resulting in 20% of labeled nuclei after 28 h (Fig. 1A). Insulin (60 ng/ml) which is non-mitogenic in these cells [17] enhances the stimulation of $PGF_{2\alpha}$, by increasing the labeled index up to 50%. TM (50–1000 ng/ml) alone or with insulin had no effect on the initiation of DNA synthesis but when added with $PGF_{2\alpha}$ or $PGF_{2\alpha}$ plus insulin it inhibited the mitogenic response (Fig. 1A).

TM (250 ng/ml) added at different times after $PGF_{2\alpha}$ alone or with insulin markedly inhibits the initiation of DNA synthesis only within the first 2 h of mitogenic induction. Addition of TM at later times resulted in a progressive loss of its effect (Fig. 1B). It disappeared when TM was added at 8–13 h after stimulation, and the labeling index obtained was similar to that observed in the absence of TM (Fig. 1B).

Stimulation of Swiss 3T3 cells by $PGF_{2\alpha}$ (300 ng/ml) increases the value of the rate constant K for cellular entry into S phase after a lag of 15 h (Fig. 2A). Insulin only potentiates the effect of $PGF_{2\alpha}$ by increasing the value of K. TM (250 ng/ml) added with $PGF_{2\alpha}$ alone or with insulin reduced the value of K without changing the length of the lag phase (Fig. 2A,B). In contrast, TM added at 8 or at 13 h after stimulation did not alter the rate of entry into S phase (Fig. 2A,B).

3.3. *Effect of tunicamycin on [^{14}C]glucosamine, [^{3}H]mannose and [^{3}H]leucine incorporation*

The effect of $PGF_{2\alpha}$ minus or plus insulin in the incor-

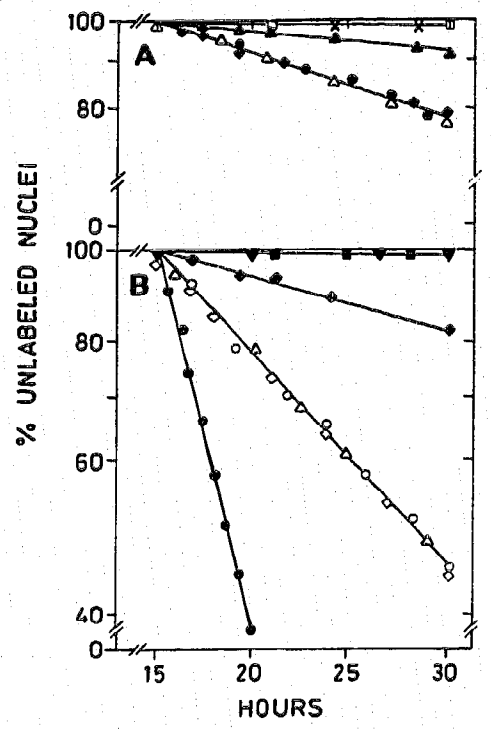

Fig. 2. Kinetics of entry into S phase stimulated by $PGF_{2\alpha}$ (300 ng/ml) without or with insulin (60 ng/ml) and in the absence of presence of TM (250 ng/ml). (A) (×), no addition (K = 0.05); (□), insulin (K = 0.06); (△), $PGF_{2\alpha}$ (K = 1.3); (▲), $PGF_{2\alpha}$ plus TM added at 0 h (K = 0.3); (◆), $PGF_{2\alpha}$ plus TM added at 8 h (K = 1.4); (●), $PGF_{2\alpha}$ plus TM added at 13 h (K = 1.5). (B) (▼), TM (K = 0.05); (■), TM plus insulin (K = 0.05); (△), $PGF_{2\alpha}$ with insulin (K = 5.3); (◆), $PGF_{2\alpha}$ with insulin and TM added at 0 h (K = 1.2); (◇), $PGF_{2\alpha}$ plus insulin and TM added at 8 h (K = 5.4); (○), $PGF_{2\alpha}$ with insulin and TM added at 13 h (K = 5.4); (●), fetal calf serum (K = 24.0). K values are given in 10^{-2} h. In all cases the duration of the lag phase was 15 h. Labeling procedures as in Fig. 1.

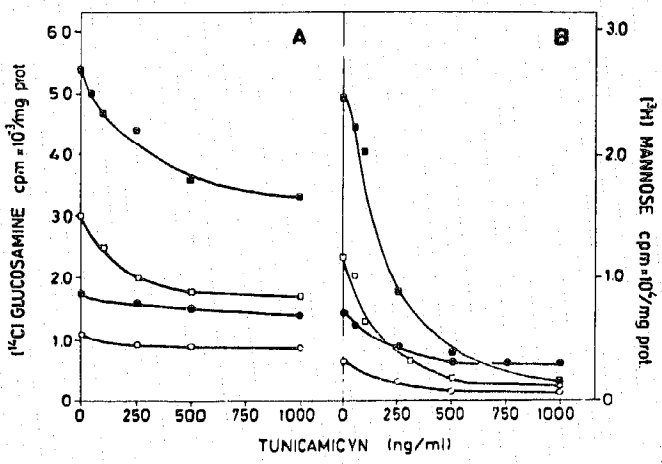

Fig. 3. Efect of TM [^{14}C]glucosamine and [^{3}H]mannose incorporation stimulated by $PGF_{2\alpha}$ (300 ng/ml) alone or with insulin (60 ng/ml). (□), $PGF_{2\alpha}$ plus TM; (■), $PGF_{2\alpha}$ plus insulin and TM; (●), insulin plus TM; (○), TM. Cells were radioactively labeled as indicated in Materials and Methods.

Table I

Comparison of TM effect on DNA synthesis, [^{3}H]mannose and [^{3}H]-leucine incorporation at different times of the lag phase upon stimulation by $PGF_{2\alpha}$ minus or plus insulin

Additions	A Labeled nuclei (%)	B [^{3}H]Mannose cpm/mg prot. ($\times 10^4$)	C [^{3}H]Leucine cpm/mg prot. ($\times 10^4$)
none	0.5	1.4	8.4
$PGF_{2\alpha}$	22.6	8.4	13.3
$PGF_{2\alpha}$ + TM	22.9	2.9	12.9
$PGF_{2\alpha}$ + insulin	55.1	12.6	15.6
$PGF_{2\alpha}$ + insulin + TM	54.3	4.3	15.7

Cells were stimulated by $PGF_{2\alpha}$ (300 ng/ml) without or with insulin (50 ng/ml). TM (250 ng/ml) was added at different times after stimulation: (A) initiation of DNA synthesis from 9 to 28 h; (B) incorporation of [^{3}H]mannose from 9 to 14 h; (C) [^{3}H]leucine incorporation, from 0 to 5 h. Measurements were as indicated in Materials and Methods.

poration of [^{14}C]glucosamine and [^{3}H]mannose into total cellular proteins is shown in Fig. 3. Addition of $PGF_{2\alpha}$ (300 ng/ml) increases the incorporation of [^{14}C]glucosamine and [^{3}H]mannose into acid-precipitable material. Insulin had a small effect on the incorporation of these sugars but together with $PGF_{2\alpha}$ enhances it. TM added at 50–1000 ng/ml reduces the incorporation of either [^{14}C]glucosamine and [^{3}H]mannose stimulated by $PGF_{2\alpha}$, insulin or $PGF_{2\alpha}$ with insulin (Fig. 3). Also TM (250 ng/ml) added from 9 to 14 h after $PGF_{2\alpha}$, minus or plus insulin inhibited the incorporation of [^{3}H]mannose, without affecting the initiation of DNA synthesis. In contrast, TM addition from 0 to 5 h to stimulated cells did not block [^{3}H]leucine incorporation into cellular proteins (Table I).

4. DISCUSSION

Previous findings indicated that *N'*-glycoproteins synthesis plays an important role in the control of cell division [11,14]. The division of Burkitt lymphoma cells can be arrested by TM in G_0G_1 phase of the cell cycle [11]. TM removal enables these cells to synthesize three major *N'*-glycoproteins prior to their entry into S phase [11]. Also TM reduces the proliferation and DNA polymerase α_2 activity in neuroblastoma cells [20]. Other results show that division of exponentially growing Swiss 3T3 cells can be reversibly blocked by TM [10]. In contrast, TM did not affect the division of polyoma 3T3 and SV40 W138 transformed cells, but it caused cytotoxicity and changes in their agglutinability [10]. In addition, it has been shown that TM inhibits the initiation of DNA synthesis in quiescent Swiss 3T3 cells stimulated by serum [14].

Other evidence reveals that TM treatment of a variety of cells decreases the presence and/or alters the properties of many *N'*-glycoproteins, some of them due to increased sensitivity to proteolytic degradation [21]. This includes surface membrane receptors for growth factors [22], hormones [7,8] or neurotransmitters [21], as well as fibronectin [23] or proteins participating in cell fusion [9].

Here we have shown that TM inhibits the initiation of DNA synthesis in Swiss 3T3 cells stimulated by $PGF_{2\alpha}$ alone or with insulin. The effect of TM is accompanied by a decrease of [^{14}C]glucosamine and [^{3}H]mannose but not [^{3}H]leucine incorporation into total cell proteins indicating that it is probably due to the blockage of the *N'*-glycosylation process and not of protein synthesis. Furthermore, TM inhibits the rate of entry into S phase only when added within the first 8 h of the lag phase regardless of blocking sugar incorporation at any time after stimulation. Thus it appears that *N'*-glycoprotein synthesis early in the lag phase is a crucial event to regulate the initiation of DNA synthesis induced by $PGF_{2\alpha}$ and possibly by other mitogens. The identification of these *N'*-glycoproteins, is the framework of our future research.

Acknowledgements: We thank Dr. J.J. Cazzulo for helpful criticisms on the manuscript. This research was supported by grants from the Association International for Cancer Research, United Kingdom, Plus Petrol S.A. and CONICET, Argentina. A.G.E. and M.G. are fellows, and L.J.A. is Principal Investigator of CONICET.

REFERENCES

[1] Pardee, A.B., Dubrow, R., Hamlin, J.L. and Kletzien, R.F. (1978) Annu. Rev. Biochem. 47, 715–750.
[2] Jimenez de Asua, L., Richmond, K.M.V., Otto, A.M., Kubler, A.M., O'Farrell, M.K. and Rudland, P.S. (1979) in: Cold Spring Harbor Conferences in Cell Proliferation, Hormones and Cell Culture, vol. 6 (Sato, G.H. and Ross, R. eds) pp. 408–424, Cold Spring Harbor, New York.
[3] Rozengurt, E. (1986) Science 234, 161–166.
[4] Racker, E. (1983) Biosci. Rep. 3, 507–516.
[5] Carson, D.D. and Lennarz, W.J. (1979) Proc. Natl. Acad. Sci. USA 76, 5709–5713.
[6] Grabel, L.B. and Martin, G. (1983) Dev. Biol. 95, 115–125.
[7] Kohno, K., Hiragun, A., Takatsuki, A., Tamura, G. and Mitsui, H. (1980) Biochem. Biophys. Res. Commun. 93, 8421–8429.
[8] Szecowka, J., Tai, L.R. and Goodman, H.M. (1990) Endocrinology 126, 1834–1841.
[9] Olden, K., Law, J., Hunter, V.A., Romain, R. and Parent, J.B. (1981) J. Cell Biol. 88, 199–204.
[10] Duksin, D. and Bornstein, P. (1977) Proc. Natl. Acad. Sci. USA 73, 3433–3437.
[11] Niskikawa, Y., Yamamoto, Y., Kaji, K. and Mitsui, H. (1980) Biochem. Biophys. Res. Commun. 97, 1296–1303.
[12] Ishii, S. and Volpe, J.J. (1990) J. Neurosci. Res. 26, 419–427.
[13] Jimenez de Asua, L., Clingan, D. and Rudland, P.S. (1975) Proc. Natl. Acad. Sci. USA 76, 1284–1287.
[14] Jimenez de Asua, L., Foecking, M.K. and Otto, A.M. (1983) Cell Biol. Int. Rep. 7, 551–552.
[15] Parodi, A.J. and Leloir, L.F. (1979) Biochim. Biophys. Acta 559, 1–37.
[16] Takatsuki, A., Kahno, K. and Tamura, G. (1975) Agric. Biol. Chem. 39, 2089–2091.
[17] Todaro, G. and Green, H. (1963) J. Cell. Biol. 17, 299–313.
[18] Brooks, R.F. (1975) J. Cell. Physiol. 86, 369–378.
[19] Jimenez de Asua, L., Richmond, K.M. and Otto, A.M. (1981) Proc. Natl. Acad. Sci. USA 78, 1004–1008.

[20] Bhattacharya, P. and Basu, S. (1982) Proc. Natl. Acad. Sci. USA 79, 1488–1491.
[21] Gilman, P.B., Keane, P. and Martinez, J. (1984) J. Biol. Chem. 259, 3248–3253.
[22] Soderquist, A.M. and Carpenter, G. (1984) J. Biol. Chem. 259, 12586–12594.
[23] Duskin, D. and Bornstein, P. (1979) J. Biol. Chem. 252, 955–962.