BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1997 Mar; 18 (2): 161 - 165

Staurosporine blocked normal cells at G_1/S boundary[1]

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KEY WORDS staurosporine; interphase; S phase; thymidine kinase; fibroblasts; cultured cells; stomach neoplasms; flow cytometry; phosphorylation

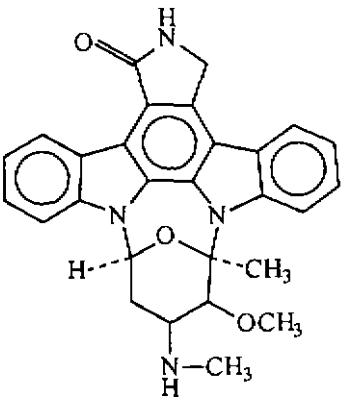
AIM: To reveal the regulating difference of G_1/S-phase transition between normal and tumor cells by using staurosporine, an unspecific kinase inhibitor. **METHODS**: Flow cytometry, Dot blot, kinase activity assay, and electrophoresis. **RESULTS**: A 18-h treatment with staurosporine (5 $\mu g \cdot L^{-1}$) blocked normal cell line 2BS cells (normal human embryonic lung fibroblast, 5 - 20 passages) in G_1 phase, decreased their thymidine kinase (TK) mRNA level and activity, and also dephosphorylated an intracellular 107 kDa protein. Meanwhile, all these effects in 2BS cells disappeared only by washing staurosporine away. Such kind of effects did not occur in tumor cell line BGC-823 cells (human stomach cancer cell). **CONCLUSION**: During the period of G_1/S-phase transition, the kinases involved are more sensitive to staurosporine in normal cells than in tumor cells.

During eukaryotic cell cycle, there are 3 key transition points: G_0/G_1, G_1/S, and G_2/M. The cell cycle control differences between tumor and normal cells appear mainly at G_1/S transition. Tumor cells can free pass this boundary so that they can be immortalized, while the normal cells are controlled very well[1].

There is a possibility that Retinoblastoma protein (RB) and its family proteins (like p107) and p53 play important roles at G_1/S-phase transition boundary by binding to the transcription factor E2F or affecting the repair of damaged DNA[2].

In order to figure out this mechanism, we chose thymidine kinase (TK) as one of our markers. Because TK is a very important kinase for DNA synthesis. It catalizes the change of thymidine nucleotides to mono-, di-, and tri-phosphates by transferring the r-phosphate from ATP to the substrate. Its enzymatic activity and mRNA level are low in G_1 phase cells, but both rise dramatically near the onset of S phase[3].

Staurosporine, a microbial alkaloid produced by Streptomyces sp, 5 $\mu g \cdot L^{-1}$ arrested normal cells (eg, foreskin cell, human embryonic fibroblast) in G_1 phase, but had no effect on tumor cells (eg, HL-60 cells)[4]. In this study, we tried to demonstrate the regulating differences at G_1/S-phase transition between normal and tumor cells.



Staurosporine

MATERIALS AND METHODS

Cell and culture conditions The human embryonic lung fibroblasts (2BS) were maintained in DMEM (Gibco) supplemented with 10 % calf bovine serum, and used within 20 passages. Human stomach cancer cells (BGC-823) were incubated in RPMI 1640 (Gibco) medium with 10 % calf bovine serum.

Staurosporine treatment 2BS or BGC-823 cells were split into 75-cm^2 flasks at a concentration of $1 \cdot 10^6$ cells/flask. These cells were kept in 37 ℃, 5 % CO_2 incubator for overnight. Staurosporine (Sigma) was dissolved with

[1] Project supported by the National Natural Science Foundation of China, № 3928004.
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Received 1996-02-28 Accepted 1996-10-25

Me_2SO and added into the cells. The Me_2SO concentration in medium was kept <0.1 %. The cells were incubated. For the release of staurosporine, the cells which have been treated were washed 3 times with serum-free medium, then incubated in fresh medium with serum.

Flow cytometric assay Cells were trypsinized, washed with cold PBS for 3 times, and fixed with 75 % cold ethanol at 4 ℃ for overnight. The cells were spun down and the pellets were washed with cold PBS 3 times. The cell pellets were resuspended with PBS containing ribonuclease A (RNase A, free DNase, Sigma) 20 mg·L^{-1} and left at 37 ℃ for 30 min. Propidium Iodide (PI, in PBS) was added to a final concentration of 50 mg·L^{-1}, and incubated at 4 ℃ for 1 - 2 h. All these samples were measured with FACS-440 (B-D Ltd).

TK expression assay Guanidine/acid phenol method was used to isolate the intracellular total RNA, then Dot blot was done and hybridized by TK cDNA probe[5].

TK activity assay Cells were harvested by tripsinization and washed with cold PBS twice and washing solution (KCl 154 mmol·L^{-1}, thymidine 16 μmol·L^{-1}, β-mercaptoethanol 3 μmol·L^{-1}) twice. The cells were resuspended with cold extracting solution (Tris· HCl 10 mmol·L^{-1}, pH 8.0, thymidine 16 μmol·L^{-1}, β-mercaptoethanol 3 mmol·L^{-1} to the concentration of 2.5×10^{10} cells·L^{-1}. The cells were frozen in ethanol-dry ice bath, then thawed at 37 ℃ for 5 cycles. The cell lysates were spun at 30 000 × g, 4 ℃ for 30 min. The supernatant was stored at −70 ℃. When analyzing TK activity, the following system was used: 30 μL reacting solution ($MgCl_2$ 15 mmol·L^{-1}, Tris· HCl 30 mmol·L^{-1}, pH 7.5, ATP 30 mmol·L^{-1}), [^{3}H]TdR 10 μL (370 kBq), cell extract 15 μL, and extracting solution 45 μL. The reaction occurred at 37 ℃ for 20 min, then was stopped by putting the tubes on ice. Spot 80 μL of each reacting mixture onto DE-81 filter. Then the filter was washed with ammonium formate 5 mmol·L^{-1} 3 times immediately, 15 min each time. The filter was rinsed once with methanol, dried and counted by Liquid Scintillation Analyzer (Packard Tri-Carb 1500).

Intracellular substrate phosphorylation assay The cells were harvested by trypsinization; washed with cold PBS for 3 times, sonificated in cold buffer A (Tris·HCl 20 mmol·L^{-1}, pH 7.5, β-mercaptoethanol 50 mmol·L^{-1}, PMSF 1 mmol·L^{-1}, egtazic acid 0.5 mmol·L^{-1}, edetic acid 2 mmol·L^{-1}, sucrose 0.33 mol·L^{-1}), then spun at 2000 × g, 4 ℃ for 10 min. Triton X-100 was added into the supernatant to 1 %. The supernatants were shaken on ice for 30 min, and spun at 12 000 × g for 30 min. The following reaction was done at 37 ℃: protein 100 μg, [γ-^{32}P]ATP 370 kBq, Tris·HCl 25 mmol·L^{-1}, pH 7.5, $MgCl_2$ 5 mmol·L^{-1}, $CaCl_2$ 0.5 mmol·L^{-1}, edetic acid 0.2 mmol·L^{-1}. The total volume was 50 μL. After 30 min, 50 μL 2 × SDS PAGE loading buffer (with β-mercaptoethanol) was added to stop the reaction. The samples were boiled for 5 min, electrophoresed with 10 % SDS-PAGE. The gels were stained with 0.25 % Coomassie bright blue R250 in 10 % acetic acid/45 % methanol/45 % H_2O, destained, dried, and exposed to X ray film at −70 ℃ for 7 d.

RESULTS

Flow cytometry analysis More than 50 samples of 2BS or BGC-823 cells showed: 2BS cells, G_1 phase 56.05 ± 0.01 %, S phase 28.12 ± 0.02 %, and G_2/M phase 16.43 ± 0.01 %; BGC-823 cells, G_1 phase 62.41 ± 0.02 %, S phase 28.26 ± 0.02 %, G_2/M phase 8.10 ± 0.03 %. After treatment with staurosporine 5 μg·L^{-1} for 18 h, 2BS cells were blocked in G_1 phase, namely 71.11 ± 0.01 % cells stayed in G_1 phase, 15.28 ± 0.03 % and 13.26 ± 0.03 % cells were in S and G_2/M phase, respectively. When these cells were released from staurosporine 5 μg · L^{-1}, they recovered back to the normal: G_1 61.05 ± 0.01 %, S 21.38 ± 0.02 %, G_2/M 18.08 ± 0.02 %. But under the same kind of treatment, BGC-823 cells basically kept the same cell cycle phase distribution: G_1 phase 62.41 ± 0.02 %, S phase 28.26 ± 0.02 %, G_2/M phase 8.10 ± 0.03 % (Fig 1).

Staurosporine 1 or 10 μg · L^{-1} showed less effect. On the other hand, staurosporine 50 or 75 μg · L^{-1}, arrested both 2BS and BGC-823 cells in G_2/M phase; in 2BS cells, G_2/M phase cell number changed to 38.21 ± 0.02 % from 16.43 ± 0.01 %; in BGC-823 cells, G_2/M phase cell number increased to 69.06 ± 0.01 % from 8.10 ± 0.03 %. This effect could not be released.

Dot blot hybridization The Dot blot hybridization with TK probe showed that staurosporine 5 μg · L^{-1} treatment caused a more dramatic decrease of TK mRNA transcription in 2BS cells than in BGC-823 cells (Fig 2).

TK activity assay TK activity was inhibited in 2BS cells when they were incubated in media containing staurosporine 5 μg · L^{-1} for 18 h: TK activity dropped from 97.47 kBq·g^{-1} to 63.10 kBq·g^{-1} ($P<0.01$). Only 67 % of the control was left. On the other hand, in BGC-823 cells, there was no change observed, their TK activity of the control group was 234.45 kBq·g^{-1}, of staurosporine

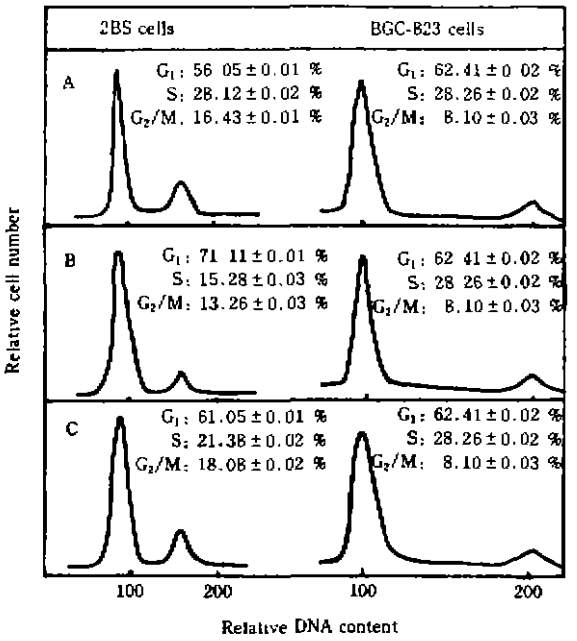

Fig 1. Flow cytometry. A) Control; B) treated with staurosporine 5 μg · L^{-1} for 18 h; C) released from staurosporine 5 μg·L^{-1} for 24 h.

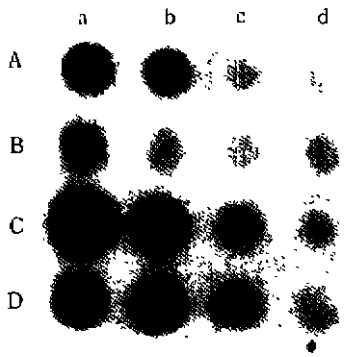

Fig 2. TK expression. A) 2BS cells; B) 2BS cells treated with staurosporine 5 μg · L^{-1} for 18 h; C) BGC-823 cells; D) BGC-823 cells treated with staurosporine 5 μg·L^{-1} for 18 h. a) RNA 10 μg; b) RNA 5 μg; c) RNA 1 μg; d) RNA 0.5 μg.

5 μg·L^{-1} group was 197.42 kBq·g^{-1}, about 84 % of the control was left.

When 2BS cells were released from staurosporine 5 μg · L^{-1}, their TK activity was restored gradually. After 24 h, it went up to 104.76 kBq · g^{-1}, which was near the original level, and no significant difference was found between the control and the released groups. TK activity of BGC-823 cells did not change from the beginning to the end (Tab 1).

Tab 1. Effect of staurosporine 5 μg·L^{-1} on TK activity in 2BS and BGC-823 cells. $n=5$, $\bar{x}\pm s$. $^aP>0.05$, $^cP<0.01$ *vs* control.

Drug time/h	Release time/h	TK Activity/kBq·g^{-1} 2BS	BGC-823
0	0	94.2±0.8	234.4±5.5
2	0	113.0±0.7	202.0±2.3
4	0	86.6±1.2	197.5±1.1
18	0	63.1±0.9^c	197.4±6.5^a
18	24	104.8±0.6^a	196.6±6.0
18	2	86.6±0.8	188.4±0.7
18	4	120.4±0.5	210.6±0.8

Intracellular protein phosphorylation test

There was no change of protein phosphorylation level in 2BS and BGC-823 cells treated with staurosporine 5 μg·L^{-1} for 1 – 4 h, but treated with staurosporine 5 μg·L^{-1} for 18 h, the phosphorylation of a 107 kDa protein in 2BS cells disappeared, and it was rephosphorylated again if the staurosporine was washed out for 24 h. BGC-823 cells behaved in a different way. Compared with the control, the phosphorylation level of this 107 kDa protein had no change after either treated with staurosporine 5 μg·L^{-1} or released from it for 24 h (Fig 3).

All these suggested the phenomenon that staurosporine 5 μg · L^{-1} treatment inhibited the phosphorylation of an intracellular 107 kDa protein was specific in 2BS cells, and such a kind of effect could be released.

DISCUSSION

Our contribution about staurosporine reported here is consistent with the Crissman's[3]. The transcription of TK is controlled by transcription factor E2F[6], and generally, E2F is negatively regulated by intracellular RB family (retinoblastoma gene product RB and p107 etc). TK activity is able to be restored when E2F-RB or E2F-p107 complex has been destroyed. In normal cells, the efficient way to break this is to phosphorylate RB or p107[7]. Considering the data we got in 2BS cells treated with staurosporine 5 μg·L^{-1} together with

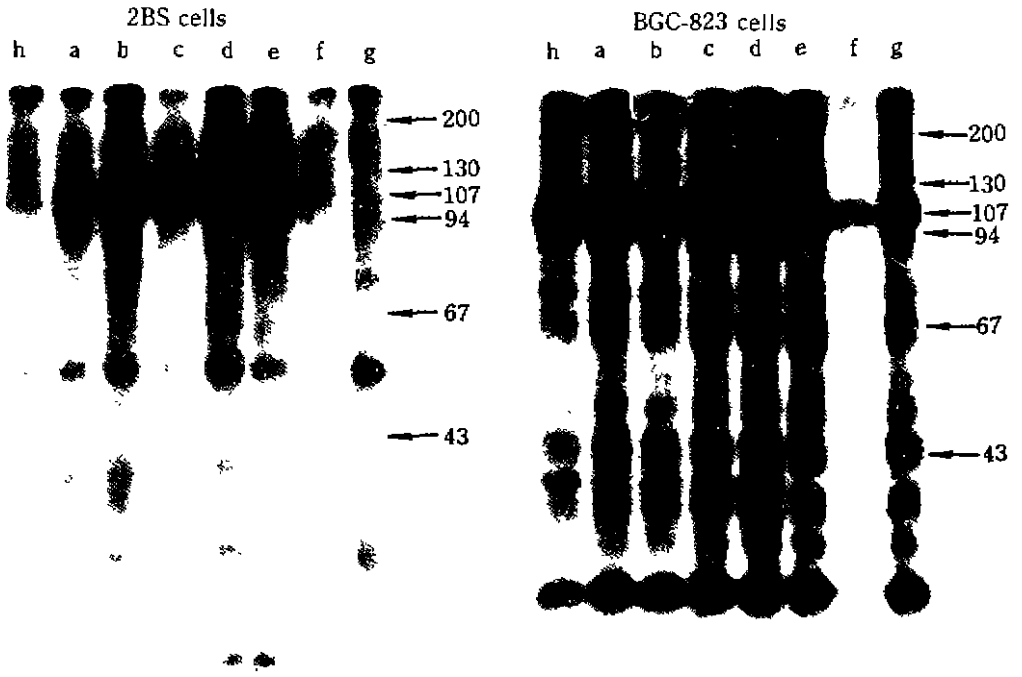

Fig 3. Intracellular substrates phosphorylation. a) Control; b) Cells treated with staurosporine 5 μg·L^{-1} for 1 h; c) 2 h; d) 3 h; e) 4 h; f & g) 18 h; h) Cells released for 24 h after 18-h treatment with staurosporine 5 μg·L^{-1}.

these facts, it is quite possible that the 107 kDa protein is p107, and kinase affected by staurosporine might be cycline A-cdk2[8-9].

On the basis of our results, we suggest that there is a reversible proliferating regulation point at G_1/S-phase boundary. This kind of regulation functions by directly affecting E2F function. In the view of the expression time differences between the genes regulated by E2F, this control point should be the result of the long process regulated by all kinds of involving kinases.

Our data confirmed that staurosporine 5 μg·L^{-1} was able to block normal cells in G_1 phase, and had nothing to do with the tumor cells. The difference of proliferation control between normal and tumor cells came from their sensitivities to staurosporine 5 μg·L^{-1} during G_1/S-phase transition. The lose of sensitivity of tumor cells to staurosporine 5 μg·L^{-1} means all their kinases involved in this regulation process get resistance to staurosporine 5 μg·L^{-1} at some extent, and this would make E2F function continuously at anytime so that the tumor cells can synthesize DNA and divide immortally.

At the same time, according to the information we got on the other several kinds of cell lines, such as NIH3T3, tsRSVNIH3T3-LA90 (33 ℃, the transformed phenotype), C3H10T1/2, and transformed C3H10T1/2, HEP2, we think staurosporine 5 μg·L^{-1} can retain many kinds of normal cells in G_1 phase, and this is very helpful for the medicine combination which is being used to cure the patients carrying cancers.

ACKNOWLEDGMENTS To Dr PANG Da-Ben and CHI Xu-Sheng for the FCM measurement and Dr ZHANG Hong-Qing for the excellent help.

REFERENCES

1 Pardee AB. G_1 events and regulation of cell proliferation. Science 1989; **246**: 603-8.

2 Hollingsworth RE Jr, Hensey CE, Lee WH. Retinoblastoma protein and the cell cycle. Curr Opin Gene Dev 1993; **3**: 55-62.

3 Kim YK, Wells S, Lan Y-FC, Lae AS. Sequences contained within the promoter of the human thymidine kinase gene can direct cell-cycle regulation of heterologous fusion genes. Proc Natl Acad Sci USA 1988; **85**: 5894-990.

4 Crissman HA, Gadbois DM, Tobey RA, Bradbury EM. Transformed mammalian cells are defficient in kinase-mediated

control of progression through the G_1 phase of the cell cycle. Proc Natl Acad Sci USA 1991; **88**: 7580 - 4.

5 Sambrook J, Fritsch EF, Maniatis F. Molecular Cloning New York: Cold Spring Harbor Laboratory Press. 1989: 7.53 - 7.55.

6 Means AL, SlAnsky JE, McMahon SL, Knuth MW, Farnham PJ. The HIP1 binding site is required for growth regulation of the dihydrofolate reductase gene promoter Mol Cell Biol 1992; **12**: 1054 - 63.

7 Nevins JR. E2F: a link between the RB tumor suprressor protein and viral oncoprotein. Science 1992; **259**: 424 - 8.

8 Elledge SJ, Richman R, Hall FL, Williams RT, Ledgson N, Harper JW CDK2 encodes a 33-kDa cyclin A-associated protein kinase and is expressed before CDC2 in the cell cycle. Proc Natl Acad Sci USA 1992; **89**: 2907 - 11;

9 Cardoso MC, Leonhardt H, Nadal-Ginard B. Reversal of terminal differentiation and control of DNA replication: cyclin A and cdk2 specially localize at subnuclear sites of DNA replication. Cell 1993; **74**: 979 - 92

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Staurosporine 阻断正常细胞于 G_1/S 时相的边界

R977

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关键词 staurosporine；核分裂间期；S期；胸腺嘧啶核苷激酶；成纤维细胞；培养的细胞；胃癌；流式细胞光度术；磷酸化

目的：通过使用非特异性激酶抑制剂 staurosporine (5 $\mu g \cdot L^{-1}$)，揭示正常与肿瘤细胞之间在 G_1/S 时相过渡阶段调控的不同。方法：流式细胞光度术，点杂交，激酶活性分析，电泳。结果：18 小时 staurosporine (5 $\mu g \cdot L^{-1}$) 的处理，可以阻断正常细胞系 2BS 细胞（人胚胎肺成纤维细胞，5 - 20 代）于 G_1 期，降低其胞内胸腺嘧啶核苷激酶（TK）的转录和活性水平，同时也去磷酸化胞内一个 107 kDa 的蛋白。在 2BS 细胞系中，所有以上现象在洗去 staurosporine 后都可以得到逆转。但是，所有以上结果在 BGC-823 细胞内（人胃癌细胞）未曾出现。结论：在 G_1/S 时相过渡阶段，正常与肿瘤细胞的调节存在差别。在正常细胞中，参与这一调节的激酶对 staurosporine (5 $\mu g \cdot L^{-1}$) 比在肿瘤细胞内的激酶敏感得多。

BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1997 Mar; **18** (2): 165 - 168

Effects of dexamethasone and ibuprofen on LPS-induced gene expression of TNF_α, IL-1β, and MIP-1α in rat lung

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KEY WORDS Wistar rats; lipopolysaccharides; tumor necrosis factor; interleukin-1; gene expression; dexamethasone; ibuprofen; respiratory distress syndrome

AIM: To study the kinetics of tumor necrosis factor α (TNF_α), interleukine-1β (IL-1β), and macrophage inflammatory protein-1α (MIP-1α) gene expression in rat lung after ip lipopolysaccharides (LPS) and the effect of dexamethasone (Dex) and ibuprofen (Ibu) on the cytokines gene expression. **METHODS**: The amount of Evans blue in lung was measured by fluorescence method. The mRNA levels of TNF_α, IL-1β, and MIP-1α in rat lung were assessed by slot blot analysis. **RESULTS**: The mRNA levels of TNF_α, IL-1β, and MIP-1α in rat lung after ip LPS increased in a dose-dependent manner, and peaked at 2, 6, and 12 h, respectively. Both Dex 50 mg $\cdot kg^{-1}$ and Ibu 90 mg $\cdot kg^{-1}$ injected at 1 h before ip LPS markedly decreased the content of Evans blue in lung at 1 h after ip LPS. After Dex or Ibu pretreatment, the peak levels of TNF_α, IL-1β, and MIP-1α mRNA decreased markedly compared with LPS alone. **CONCLUSION**: The gene expression of TNF_α, IL-1β, and MIP-1α in rat lung increased

Received 1995-08-28 Accepted 1996-06-10