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Induction of Urokinase-type Plasminogen Activator by Sodium Salicylate in a Glioblastoma Cell Strain

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Summary

Urokinase-type plasminogen activator (uPA) is an extracellular protease involved in many physiological and patological processes. In this paper its induction by sodium salicylate (NaSal) in A1235 glioblastoma cell strain is described. Maximum uPA induction was observed 24–36 h after NaSal treatment and levels of enzyme produced were 5–7 times as high as those of untreated control. uPA induction was based on the uPA promoter activation – both, 2.2kb and 5.5kb uPA promoters, containing single and two AP-1/PEA3 binding sites, respectively, were activated. NaSal also caused substantial cell growth inhibition and cell morphology changes. Curcumin and pyrrolidine dithiocarbamate (PDTC), NF-κB inhibitors, did not abrogate NaSal-induced uPA promoter activity, nor induced uPA, suggesting that NF-κB is not involved in this uPA induction. This observation was also confirmed by immunoblot analysis on IκB. Aspirin (acetylsalicylate) also activated uPA promoter and induced uPA production. The results in this paper suggest that NaSal induced uPA production is based on the uPA promoter activation through an NF-κB independent pathway.

Key words: urokinase, uPA promoter, sodium salicylate, NF-κB, *gadd45*, glioblastoma cell strain

Introduction

Urokinase plasminogen activator (uPA) is a serine protease which triggers a proteolytic cascade leading to the degradation of the extracellular matrix. It plays the central role in tissue remodelling in a number of physiological and pathological processes, including tissue repair, pregnancy, angiogenesis as well as tumor cell invasion and metastasis *(1)*. uPA is also involved in non-proteolytic signaling events leading to adhesion and chemotaxis *(2,3)*.

In vitro, uPA synthesis could be induced by multiple agents *(1)*. Transcription of the human uPA gene is promoted by a TATA box containing minimal promoter, rich in Sp1 sites and strongly activated by an enhancer located at about 2 kb upstream *(4)*. For its activation the enhancer requires cooperation between upstream combined PEA3/AP-1_A and a downstream AP-1_B site, separated by a 74 bp »cooperation mediator« (COM) region *(5, 6)*. Downstream of AP-1_B, there are also several NF-κB-like sequence binding sites *(7)*, as well as negative regulatory elements which modulate the enhancer activity, found to be dependant on the cell type *(4,8)*. Second AP-1/PEA3 element, located at ~5.5 kb could synergize with the first one in the full induction of the gene *(9)*.

Reflecting the wide spectrum of its functions, uPA expression is regulated by numerous extracellular stimuli depending also on the cell type. Transcription could be induced by growth factors, phorbol esters, cytokines, cytoskeletal reorganization and several oncogenes.

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Abbreviations: uPA, urokinase-type plasminogen activator; NaSal, sodium salicylate; PDTC, pyrrolidine dithiocarbamate

There are several signaling pathways known to be involved in the uPA gene induction: ras-ERK1 signaling *(10)*, protein kinase C and protein kinase A activation *(11)*, cytoskeletal reorganization causing the activation of the proximal PEA3/AP-1 site of the promoter *(12)*. In the NIH 3T3 cells, both ERK and JNK activity are necessary for the uPA+ phenotype *(13)*. Most of the induction pathways include transcriptional factors binding to the proximal PEA3/AP-1 enhancer: c-Jun homodimers and heterodimers *(12,14)*. Distal AP-1/PEA3 site could bind c-Fos-c-Jun heterodimers and PEA3 site members of the Ets family of transcriptional factors *(10,15)*.

In this work the induction of uPA production by sodium salicylate (NaSal) and aspirin (acetylsalicylate) in A1235 glioblastoma cell strain is described. The mechanism of action of NaSal and aspirin are only partially known. Aspirin inhibits the activity of cyclooxygenase (COX), a key enzyme in biosynthesis of proinflammatory prostaglandins *(16)*. NaSal is produced in the cell from aspirin and, although a weak COX inhibitor, it also exerts antiinflammatory action *(17)*. It inhibits TNF-induced NF-κB *(18)*, as well as UV- and tumour promoter-induced AP-1 *(19,20)*, activates p38 and JNK, depending on the cell type *(21,22)*, and can cause apoptosis *(21)*. With these considerations in mind, the possible pathways involved in NaSal and aspirin mediated uPA induction were examined.

Material and Methods

Plasmids and cells. Plasmid uPA-CAT2212, containing human 2.2 kb uPA promoter fused to the CAT gene, was generously provided by Dr. F. Blasi (DIBIT, Milan, Italy). Plasmid 5.5kb-uPACAT, containing human 5.5 kb uPA promoter fused to the CAT gene, was obtained from Dr. Y. Nagamine (Fridrich Miescher Institute, Basel, Switzerland). Plasmid pSV2neo contained geneticin resistance gene. All the plasmids were purified by alkaline lysis method as described previously *(23)*.

Human glioblastoma cell line A1235, obtained from S. A. Aaronson (NCI, NIH, Bethesda, MD), and its transfectants were cultivated in DMEM (Sigma Chemicals, St. Louis, MO), and supplemented with 10 % fetal bovine serum (Gibco Life Technologies, UK) and antibiotics at 37 °C and 5 % CO_2. Cell counts were performed by Coulter Counter.

Antibody. IκB-α (FL) polyclonal antibody (sc-847, Santa Cruz Biotechnology, CA) was a gift from Dr. M. Giacca (ICGEB, Trieste).

Chemicals and enzymes. Curcumin, sodium salicylate, aspirin, and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma, human urokinase from Calbiochem and human plasminogen from KabiVitrum (Stockholm, Sweden). D-threo-[dichloroacetyl-1-^{14}C]chloramphenicol was purchased from Amersham (UK) and Acetyl-coenzime A from Boehringer Mannheim (Germany).

Transfection and CAT assay

A1235 cells were transfected by calcium phosphate coprecipitation as described previously *(23)*. Briefly, the exponentially growing cells ($2 10^5$ cells per 25 cm^2 tissue culture flask) were cotransfected with 10 μg of either 2.2 kb uPA-CAT2212 or 5.5kb uPACAT plasmid DNA and 2 μg of pSV2neo. After 4 h of incubation, cells were washed free of calcium phosphate precipitate and incubated in fresh medium for 48 h. Cells were then split and subsequently grown in selective medium containing 500 μg/mL of geneticin; colonies were isolated after 2–3 weeks. Transformed cells were treated with different concentrations of sodium salicylate for 24 h, and incubated in drug-free medium for an additional 24 h. Cell extracts were then prepared and assayed for the chloramphenicol acetyltransferase (CAT) activity according to a standard procedure *(23)*. Namely, 10 μg of cellular extract was incubated with 267 μM acetyl-coenzyme A and 0.5 μCi D-threo-[dichloroacetyl-1-^{14}C]chloramphenicol for 1 h at 37 °C, and after ethyl acetate extraction separated by thin layer chromatography. After exposure, bands on X ray film were analysed by Image Master VSD Software (Pharmacia Biotech, Uppsala, Sweden).

Plasminogen activator analysis

Exponentially growing A1235 cells were treated with different concentrations of NaSal for different periods of time, up to 24 h. Six hours before the end of the experiment cells were incubated in serum-depleted medium. The conditioned media were then collected and cell lysates prepared as previously described *(24)*. Samples were assayed for uPA activity using radial caseinolysis on 1 % agarose plates containing 2.5 % nonfat powdered milk, 0.1 % sodium azide and 5 μg/mL of human plasminogen. 5 μL of conditioned media was added to the wells made on the agarose plate, diameters of clear areas measured after incubation at 37 °C for 24 h and uPA activity estimated by interpolation from a calibrated curve of human urokinase *(25,26)*.

PAGE and immunoblot analysis

Total cell extracts were prepared using lysis buffer (50 mM Tris HCl pH=8.0, 150 mM NaCl, 0.02 % Na azide, 100 μg/mL phenylmethylsulfonylfluoride (PMSF), 1 % Triton X-100) and protein concentration was determined according to Bradford *(21)*. Protein samples were mixed with the loading buffer, boiled at 95 °C for 5 min, loaded on a 12 % SDS-PA gel and electrophoresed as described previously *(23)*. Proteins were then transferred onto nitrocellulose membrane (Hybond C, Amersham) in transfer buffer (25 mM TrisHCl pH=8.3, 192 mM glycin, 20 % methanol), employing Hoefer (Amersham-Pharmacia) transblot apparatus. Blots were blocked overnight in PBST (0.1 % Tween-20 in phosphate buffered saline) containing 5 % nonfat dried milk. Membranes were incubated with primary antibodies to IκB-α for 1 h. Alkaline phosphatase goat anti-rabbit IgG (Oncogene Science, Cambridge, USA) was used as a secondary antibody and the bands were detected by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) staining.

uPA detection by SDS-PAGE

Conditioned supernatants from the treated cells were collected as described above and concentrated by centrifugation in Centricon-30 tubes (Amicon, MA, USA). Samples were run on 12 % polyacrylamide gel

under nondenaturing conditions. After electrophoresis, the gel was incubated in 2.5 % Triton X-100 for 2–3 h and rinsed in water for 1 h. Gel was then incubated on the casein underlayer (1 % agarose, 3–4 % casein, Na-azide, 25 μg/mL human plasminogen) for 24 h on 37 °C, when lysis zones coresponding urokinase bands could be seen *(24).*

Results

Sodium salycilate influences cell growth

As shown on Fig. 1, A1235 cells treated for 24 h with 6.6 mM NaSal grew at nearly normal rate, 16.6 mM NaSal suppressed the cell growth, the duplication time being increased from 24 h up to 60 h, but without obvious cell dying. Cells treated for 24 h with higher (33.3 mM) NaSal concentrations, or for longer time period with 16.6 mM NaSal, did not resume the growth following the drug removal and went to apoptosis (data not shown).

Cells treated with 16.6 mM or higher concentrations of NaSal also appeared morphologically different (Fig. 2).

Sodium salicylate induces uPA production in A1235 cells

The results of the experiments, in which the effect of NaSal on the production of extracellular uPA in A1235 cells was examined, are illustrated in Fig. 3 and Table 1. Cells were treated with 6.6, 16.6 or 26.6 mM NaSal for up to 24 h and uPA activity in the conditioned medium examined by caseinolysis. As shown in Fig 3, 6.6 mM NaSal induced 3 fold control level of uPA production measured 6 h after 24-hour exposure to the drug. A maximum of about 6-fold increase of uPA production was observed with 16.6 and 26.6 mM, and at these concentrations there was no significant dose-dependency. Induction was observed no matter whether or not cells were incubated in the presence of NaSal in the last 6 h (data not shown).

As 16.6 mM NaSal appeared the least toxic for the cells with maximal uPA induction, the kinetics of production was examined. As shown in Table 1, cells started to produce uPA after a 12 h exposure to the drug, but maximal production appeared between 24 and 36 h after the beginning of the experiment. After 48 h uPA production started to decrease.

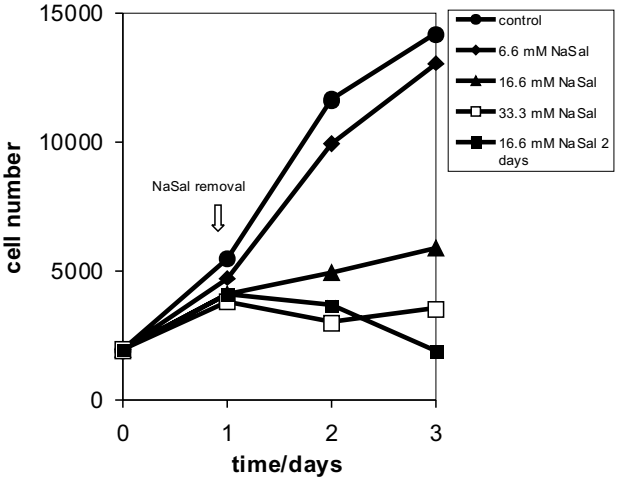

Fig. 1. Effect of NaSal on the growth of A1235 cells; cells were treated with indicated concentrations of NaSal for 24 h and incubated in the drug-free medium until the end of experiment. Cell number was determined electronically at daily intervals

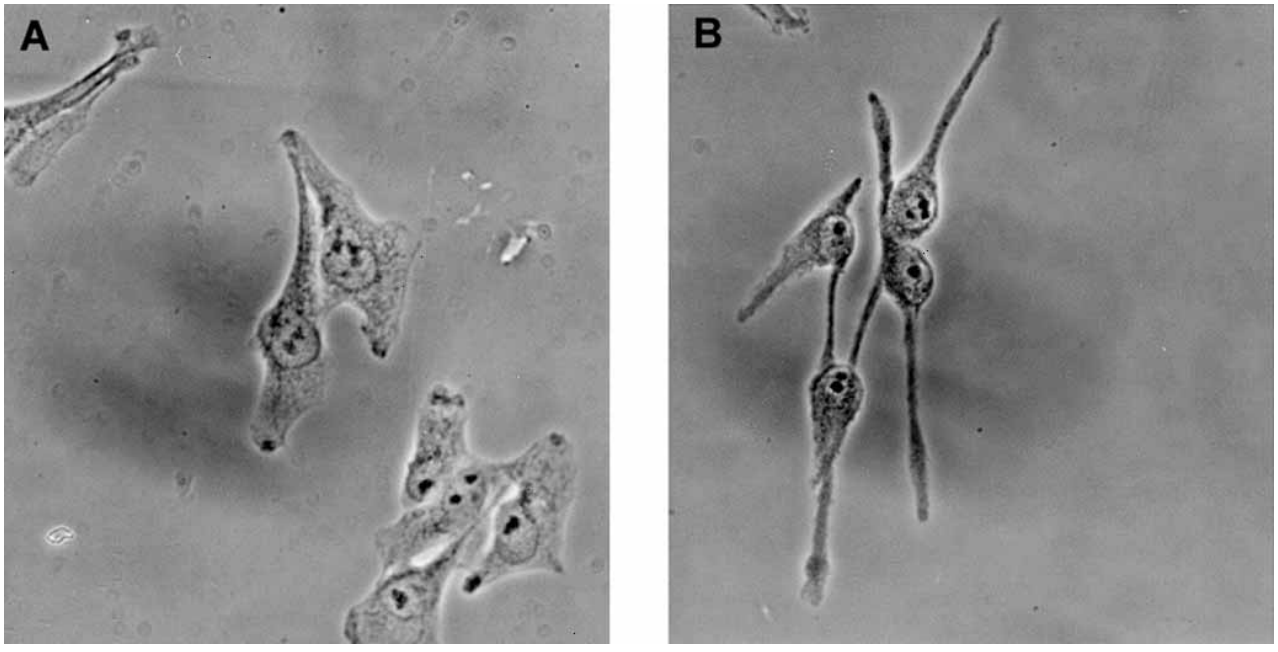

Fig. 2. Effect of NaSal on the morphology of A1235 cells; cells were treated with 16.6 mM NaSal for 24 h, fixed in methanol and photographed under phase-contrast Opton microscope (400x mag.). **A)** control cells; **B)** treated cells

Table 1. Induction of uPA in A1235 cells after treatment with 16.6 mM NaSal

Time (h)	Enhancement factor (mean ±SD)	No. of experiments
6[1]	1.2 ± 0.2	2
12[1]	2.5 ± 0.8	4
24[1]	6.1 ± 0.6	4
24 + 6[2]	5.9 ± 2.7	12
24 + 12[2]	6.8 ± 3.4	4
24 + 24[2]	2.0 ± 0.5	6

[1] Cells treated with NaSal for 6, 12 and 24 h
[2] Cells treated with NaSal for 24 h and left for additional 6, 12 and 24 h in the medium without the drug

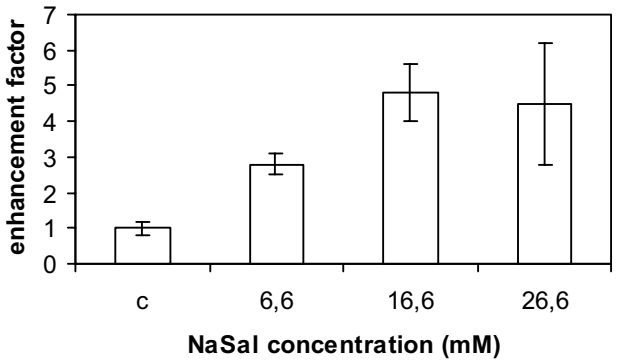

Fig. 3. NaSal induces uPA production in A1235 cells; cells were treated with indicated concentrations of NaSal for 24 h and left in the medium without serum and NaSal for 6 h (representative experiment). Extracellular uPA was determined in the conditioned medium by caseinolysis (Materials and Methods)

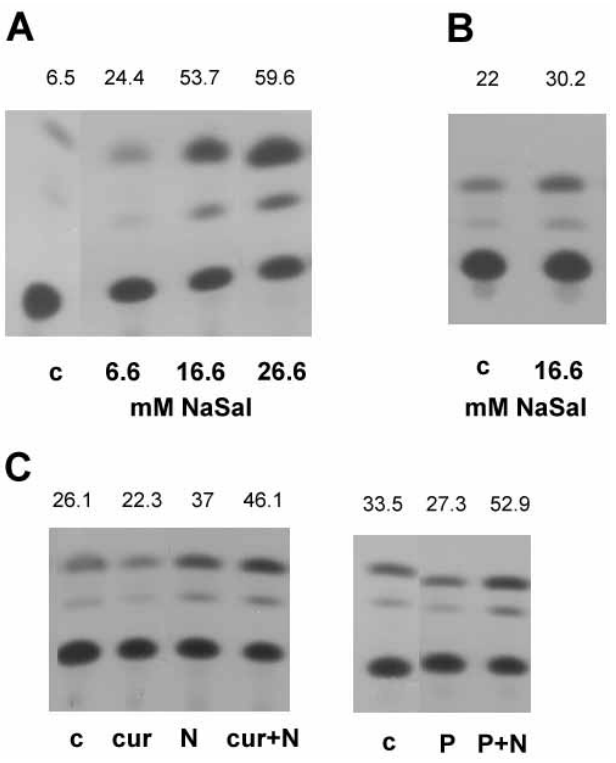

Fig. 4. NaSal enhances uPA promoter activity; A1235 cells were permanently transfected with either 5.5 kb (**A**) or 2.2 kb uPA promoter-CAT reporter plasmid (**B,C**). For each experiment cells were treated with NaSal for 24 h (A: 6.6 - 26.6 mM NaSal, B and C: 16.6 mM NaSal), washed and incubated for additional 24 h in the drug-free medium, while cell extracts were collected and CAT assay performed. To determine the effects of inhibitors (**C**), cells were exposed to curcumin or PDTC 30 min before the addition of NaSal and throughout the experiment. Numbers above figures represent percentage of chloramphenicol conversion (CAT activity) obtained by densitometry
c, control; N, 16.6 mM NaSal; cur, 25 μM curcumin; N+cur, 16.6 mM NaSal and 25 μM curcumin; P, 2.5 μM PDTC; N+P, 16.6 mM NaSal and 2.5 μM PDTC.

Sodium salicylate induces uPA promoter activity

To determine whether NaSal-induced uPA production is based on the uPA promoter activation, uPA promoter activity from NaSal-treated A1235 cells permanently transfected with either 2.2 or 5.5 kb human uPA promoter/CAT reporter plasmid was measured. As shown in Fig. 4A, A1235 cells transfected with the 5.5 kb uPA promoter/CAT (containing both PEA3/AP-1 sites) and treated with 16.6 or 26.6 mM NaSal exerted several-fold increase over uPA promoter basal activity. Likewise, A1235 cells transfected with the 2.2 kb uPA promoter/CAT plasmid showed enhanced promoter activity in the presence of 16.6 mM NaSal (Fig. 4B). These results suggest that the 2.2 kb uPA promoter containing only proximal enhancer sites, including a proximal AP-1/PEA3 site, is sufficient for its activation by NaSal. The 2.2 kb promoter activation seems to be less efective, but that is probaby due to clonal differences (maybe in their basal uPA activity) since the permanently transfected cell lines obtained from different clones were used permanently.

Since the proximal part of the uPA promoter contains NF-κB and AP-1 binding sites, cells transfected with uPA 2.2 kb promoter/CAT plasmid were treated with curcumin, an AP-1 and NF-κB inhibitor (*27*), and assayed for the CAT activity. As shown in Fig. 4C, curcumin decreased uPA promoter basal activity, but it did not significantly affect its NaSal-induced activity. In the parallel experiment with PDTC, a known AP-1 activator and NF-κB inhibitor (*28*), it neither induced uPA promoter nor abrogated its NaSal-induced activity.

Sodium salicylate does not inhibit NF-κB in A1235 cells

NaSal is known to inhibit TNF-α-mediated-NFκB induction by inhibiting IκB kinase mediated phosphorylation of IκB, its degradation and subsequent NF-κB activation (*29*). A possible involvement of NF-κB in NaSal mediated uPA induction was questioned. To test it, A1235 cells were treated with either 16.6 or 33.3 mM NaSal for 1, 6 or 24 h, the latter cultures also being incubated with drug-free medium for additional 6 h, and then collected for immunoblot analysis with IκB-α antibody. As shown in Fig. 5, all of NaSal-treated cultures showed control levels of IκB-α protein, suggesting that NF-κB is probably not involved in NaSal mediated uPA induction.

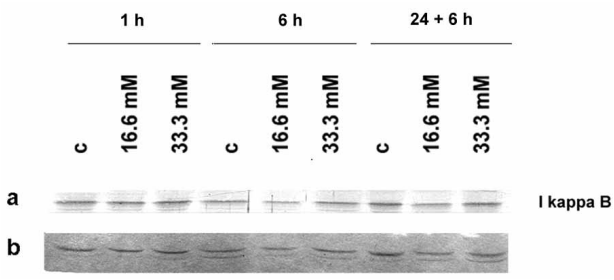

Fig. 5. Effect of NaSal on IκB expression in A1235 cells; cell monolayers were treated with indicated concentrations of NaSal for 1, 6, and 24 h, the latter being incubated in the drug-free medium for additional 6 h. At the end of incubation periods cell extracts were prepared and analysed by Western blotting with IκB antibody. **A)** membrane immunoblotted with Ikb antibody and stained with alkaline phosphatase-NBT-BCIP system; **B)** Coomassie blue stained gel

Aspirin induces urokinase in A1235 cell line

Since aspirin is converted to salicylate *in vivo*, it was of interest to determine its effect on the uPA production. Cells were parallelly treated with 20 mM NaSal and 20 mM aspirin for 24 h, washed and incubated in the medium without serum for additional 10 h. Conditioned media were collected, concentrated, electrophoresed and analysed by zimography using casein underlay. Results are shown in Fig. 6A. Both NaSal and aspirin treated samples exerted strong lysis zones corresponding to 50 kDa and 33 kDa urokinase (*1*), although aspirin effect was weaker. Fig. 6B shows results of experiments in which the effect of aspirin (20 mM) on the induction of uPA promoter in the permanently trasfected A1235 cells with the 5.5 kb uPA promoter/CAT plasmid was examined. Aspirin strongy activated uPA promoter, although less then NaSal.

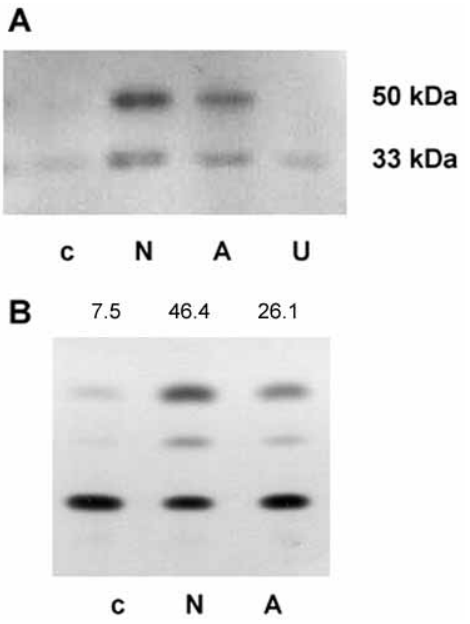

Fig. 6. Aspirin induces uPA; A) uPA production: cells were treated with 20 mM NaSal and 20 mM aspirin for 24 h, and incubated in the medium without serum for additional 10 h when conditioned medium was collected, concentrated and analysed by PAGE. Cell extracts were also prepared and protein concentration determined. The quantity of the supernatants put on the gel was normalised. As a positive control, 0.01 U/μL urokinase was used. The gel was incubated at 37 °C for 24 h on the second indicator gel containing human plasminogen. Numbers above the gel represent relative intensity of the bands, as determined by densitometry
B) uPA promoter activation; A1235 cells permanently transfected with 5.5 kb uPA promoter-CAT plasmid were treated with 20 mM NaSal and 20 mM aspirin for 24 h, incubated for additional 24 h in the drug-free medium and collected for the CAT assay. Numbers above figures represent percentage of chloramphenicol conversion (CAT activity) obtained by densitometry
c, control; N, 20 mM NaSal; A, 20 mM aspirin; U, 0.01 U/μL urokinase

Discussion

The data presented in this paper show that NaSal induces high levels of uPA production in A1235 glioblastoma cell strain and that this induction was based on the uPA promoter activation. Both the 2.2 and 5.5 kb long promoters were responsive, suggesting that proximal enhancer sites of the 2.2 kb uPA promoter were sufficient for its activation by NaSal.

The proximal part of uPA promoter has a number of transcriptional factor-binding sites, the most important being those for AP-1 and NF-κB. AP-1 induction through PEA3/AP-1 enhancer site was reported to be one of the most common pathways of uPA induction (*6,10,30*). uPA promoter can also be activated by NF-κB-like transcription factors (*7*).

In the context of the presented experiments, the following aspects of NaSal action are important: it inhibits TNF-α induced NF-κB (*18*) by inhibiting IκB kinase mediated phosphorylation of IκB, its degradation and subsequent NF-κB activation (*29*). It also inhibits UV and tumour promoter induced AP-1 (*20*). With these considerations in mind, NaSal mediated uPA induction pathway using different inhibitors was examined. It was found that cell growth arrested alone caused by serum deprivation or by hydroxyurea did not activate uPA promoter. Serum deprivation, as a matter of fact, inhibited the basal level of uPA production (data not shown). Curcumin, a known inhibitor of AP-1 and NF-κB (*27,31*), decreased basal levels of uPA promoter activity. It did not, however, abrogate NaSal-induced uPA promoter activity. Likewise, another NFκB inhibitor, PDTC, which unlike curcumin up-regulates AP-1 activity (*28*), neither activated uPA promoter nor abrogated NaSal-induced uPA production. Since PDTC and curcumin effects are not specific for just one transcription factor and probably act on the more upstream levels (*i. e.* PDTC is an antioxidant) (*28*), immunoblot analysis of IκB was performed. Control levels of IκB protein observed in drug treated cells also suggest that NF-κB is probably not involved in these pathways. A comparable uPA production in the presence and after removal of NaSal from culture medium, after a 24 h-long treatment by NaSal was detected. This would not be the case if NaSal inhibition of NF-κB was responsible for uPA induction. The lack of NF-κB involvement in NaSal mediated uPA induction is not surprising, since NaSal is known to in-

hibit just certain pathways of NF-κB induction (TNF-α activated and not IL-1 activated) (*18*,*32*), and therefore, it could act upstream, close to the activated membrane TNF receptor (*21*).

NaSal is known to induce apoptosis by p38 and caspase activation, as well as by NF-kB inhibition in certain cell lines (*21*,*33*). On the other hand, it has been suggested that a stress mediated enhancement of extracellular uPA production could be associated with the loss of cell viability and apoptosis (*34*). This could be true for some apoptosis inducing agents, but not for apoptosis in general, even in the same cell type. For instance, in these experiments A1235 cells were treated with different agents, and many of them caused apoptosis (*e.g.* high concentrations of PDTC), but did not induce uPA production. Futhermore, uPA induction was observed under conditions of cell growth suppression, but under highly cytotoxic conditions uPA production was decreased (data not shown), suggesting that NaSal-enhanced uPA production is not directly associated with the cell death.

NaSal treated cells acquire a characteristic shape (Fig. 3), suggesting that morphological changes could be connected with cytoskeletal reorganisation, known to induce uPA in certain cell types (*12*).

Experiments with aspirin confirmed those with NaSal. Aspirin, being widely used as nonsteroidal antiinflammatory drug, is an intracellular precursor of salicylate. It inhibits the activity of cyclooxygenase, a key enzyme in biosynthesis of proinflammatory prostaglandines (*16*). On the contrary, NaSal does not influence purified COX, yet it still supresses prostaglandine synthesis (*17*). All these data indicate that both NaSal and aspirin can act on several levels in the cell, possibly through the activation of some specific transcriptional factors as well. NF-κB independent mechanism of NaSal action was found in nitric oxide synthase (*35*) and cyclooxygenase 2 gene inhibition (*17*). Lower promoter activation and uPA production obtained by aspirin treated cells, in comparison with NaSal, could be the effect of the lower cellular uptake of aspirin due to higher pH of the solution (as this weak organic acid is dissolved in high pH in the form of Na-salt) (*36*). Aspirin induced uPA in the endothelial or blood cells could take a part in the prevention of cardiovascular diseases (*20*).

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Indukcija urokinaznog tipa plazminogen-aktivatora natrijevim salicilatom u glioblastomskoj staničnoj liniji A1235

Sažetak

Urokinazni tip plazminogenskog aktivatora (uPA) je ekstracelularna proteaza koja sudjeluje u mnogim fiziološkim i patološkim procesima. U ovom je radu opisana indukcija uPA natrijevim salicilatom (NaSal) u stanicama glioblastoma A1235. Maksimalna urokinazna indukcija primijećena je tijekom 24–36 sati nakon obradbe s NaSal, a razina proizvedenog enzima bila je 5–7 puta veća nego u kontrolnom uzorku. Indukcija uPA temeljila se na aktivaciji promotora uPA gena: aktivirana su oba uPA promotora od 2,2 i 5,5 kb s jednim ili dva AP-1/PEA3 mjesta vezanja. NaSal je također uzrokovao veću inhibiciju staničnog rasta i promjene stanične morfologije. NF-κB inhibitori, kurkumin i PDTC, nisu spriječili aktivnost uPA promotora natrijevim salicilatom, a niti su inducirali uPA, pokazujući da NF-κB ne sudjeluje u indukciji uPA. To je potvrđeno »Western blot« analizom na IκB. Aspirin, acetilsalicilat, također aktivira uPA promotor i inducira proizvodnju urokinaze. Rezultati upućuju na to da natrijev salicilat i aspirin induciraju uPA proizvodnju aktivacijom uPA promotora putem neovisnim o NF-κB.