The role of NUPR1 in lymphocyte proliferation and apoptosis

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Abstract: Nuclear protein 1 (NUPR1) is a transcription cofactor that senses stressful conditions and modulates cellular response by promoting or inhibiting apoptosis. NUPR1 is usually highly expressed in tumor cells where it enables them to adapt and resist environmental stress or chemotherapeutic compounds. NUPR1 can be involved in cell proliferation. Data about the involvement of NUPR1 in the proliferation and apoptosis of lymphocytes are scarce. Therefore, in this study we focused on the role of NUPR1 in lymphocyte physiology and found that NUPR1 might be involved in the initiation of their proliferation. Lymphocytes were isolated from the cervical lymph nodes of C57BL/6 mice. NUPR1 expression subsided 24 h after the induction of proliferation by a mitogen. Also, stressful conditions after cell isolation led to increased NUPR1 mRNA and protein expression *in vitro* that coincided with cell apoptosis. Similarly, apoptosis induction by staurosporine, a broad-range protein kinase inhibitor, led to increased NUPR1 expression. In addition, NUPR1 inhibition by small-interfering RNA prevented the staurosporine-induced apoptosis (judging from decreased caspase activity) in the whole cell population of cervical lymph nodes. However, NUPR1 absence was irrelevant to the induction of apoptosis in $CD3^+$ T lymphocytes, suggesting that NUPR1 is probably a mediator of apoptosis in other immune cell populations within the lymph node, such as B lymphocytes. In conclusion, our results suggest that NUPR1 is important for the initiation of lymphocyte cell division and for the apoptotic process of non-T cells during stressful conditions.

Key words: apoptosis; cervical lymph node; apoptosis; lymphocyte; nuclear protein 1; proliferation

INTRODUCTION

Nuclear protein 1 (NUPR1) is a small chromatin protein that senses stress signals from the environment and enables cells to adapt and to resist apoptosis. NUPR1 is a protein of 8 kDa (hence its other name, p8) that can interfere with histone modifications and bind to gene promoters, thereby modulating gene expression. NUPR1 is important for the resistance of tumor cells to apoptosis induction since it specifically leads to the transactivation of the immediate early response 3 (*Ier3*) gene in pancreatic cancer cells that protects cells from Fas- or tumor necrosis factor (TNF)-induced apoptosis [1]. Also, NUPR1 binds thymosin and interferes with the apoptotic cascade in HeLa cells. The absence of NUPR1 renders HeLa cells susceptible to staurosporine-induced apoptosis [2]. Therefore, it is not surprising that elevated NUPR1 expression is found in breast cancer cells, pancreatic carcinoma cells and adenocarcinoma [3,4]. In breast cancer cells, the overexpression of NUPR1 leads to the activation of the phosphatidylinositide 3-kinase (PI3K)/Protein kinase B (PKB) (Akt) signaling pathway, cyclin-dependent kinase inhibitor 1 (CDKN1A; $p21^{Cip1}$) phosphorylation and relocalization from the nucleus to the cytoplasm that enables malignant cells to resist the action of chemotherapeutics. The involvement of NUPR1 in the malignant process is further confirmed by the fact that transforming growth factor β (TGF-β), a cytokine involved in tumorigenesis, stimulates *NUPR1* expression [5]. Obviously, this prosurvival function of

NUPR1 supports cancer progression. However, NUPR1 can have a completely opposite role in the regulation of apoptosis since it can promote autophagy-induced apoptosis via the inhibition of Akt/mammalian target of rapamycin complex-1 (mTORC1) signaling after sensing stress-induced changes in the endoplasmic reticulum [6, 7]. NUPR1 might also have a role in the control of cell proliferation since its complete deletion increases pancreatic β-cell proliferation [8]. Also, NUPR1 negatively regulates the proliferation of myocytes and promotes myogenic differentiation [9,10]. As regards immune cells, it was shown that the number of all immune cells is comparable between $NUPR1^{-/-}$ and $NUPR1^{+/+}$, though the apoptosis rate under normal and inflammatory conditions is increased in the absence of NUPR1, especially in $CD3^+$ cells [11].

To date, there are no data about the role of NUPR1 in the proliferation and stress-induced apoptosis of immune cells. Therefore, in this study we investigated the correlation of NUPR1 mRNA and protein expression and mitogen-triggered immune cell proliferation or apoptosis induced either by the process of cell isolation or by staurosporine, a broad-spectrum protein kinase inhibitor.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice were bred and kept in the animal facility of Institute for Biological Research "Siniša Stanković", and their use and all experimental procedures were approved by the Ethics Committee of the Institute (App. No 01-07/15), which are in accordance with Directive 2010/63/EU. The mice were maintained under standard conditions with a 12-h day/night rhythm and fed with standard rodent chow and water.

Isolation of lymphocytes from cervical lymph nodes

C57BL/6 mice (8-12 weeks of age) were euthanized by CO_2 affixation and cervical lymph nodes were collected in tubes filled with phosphate-buffered saline (PBS). Lymph nodes were passed through 40-μm cell strainers and dispersed into a single cell suspension. After centrifugation at 500 g for 5 min, cervical lymph node cells (CLNC) were resuspended in RPMI-1640 medium containing 25 mM HEPES, 2 mM L-glutamine, 5% FCS (PAA Chemicals, Pasching, Austria), penicillin/streptomycin (Sigma-Aldrich, St. Luis, MO, USA) and 5 μM β-mercaptoethanol, counted by trypan blue exclusion test (Sigma-Aldrich) and placed in 24-well plates at 37°C in a 5% CO_2 incubator. CLNC were cultured in the media alone or in the presence of 1 μg/mL concanavalin A (ConA), or with 1 μM staurosporine (Sigma-Aldrich).

$CD3^+$ lymphocyte purification

CLNC (1×10^7) were resuspended in 5 mL of RPMI-1640 and 10% FCS. For separation of adherent and nonadherent cells, a column made of nylon wool was soaked first in 5 mL of medium (RPMI-1640 and 10% FCS) and the resuspended cells were overlaid. The column with cells was maintained for 1 h at 37°C in a 5% CO_2 incubator and nonadherent cells were eluted with warm medium (2 x 5 mL). The recovery of cells was about $2x10^6$; the purity of $CD3^+$ cells was 92.2±8.2% (determined by flow cytometry analysis).

NUPR1 inhibition by small interfering RNA (siRNA)

In a 24-well tissue culture plate, 1×10^6 CLNC were placed in 1 mL of antibiotic-free medium supplemented with 10% FCS. After overnight incubation, the cells were transfected according to the manufacturer's instructions (SantaCruz Biotechnology Inc., Santa Cruz, CA, USA). Briefly, incubation in transfection medium containing NUPR1 or control small-interfering RNA (siRNA) lasted for 6 h. The cells were then washed and placed in RPMI-1640 medium with 10% FCS and antibiotics. After overnight incubation (18 h), 1 μM staurosporine was applied for 3 h before the cells were collected and their apoptosis was measured by determining caspase activity by flow cytometry. Transfected CLNC were also challenged for

24 h with ConA and proliferation was measured by the 3-(4,5-dimethyl-2-thiazolyl)-2-5-diphenyl-2H-tetrazolium bromide (MTT) assay.

Apoptosis detection

After the above treatments, CLNC were stained with FITC-annexin V (BD Pharmingen, San Diego, CA, USA) and propidium iodide (Life Technologies Ltd, Paisley, UK) (10 ng/mL) for 10 min at room temperature in the dark in the specific annexin-binding buffer, washed in the same buffer and analyzed on a Partec CyFlowSpace flow cytometer by FlowMax software (Partec, GmbH, Münster, Germany). To detect cells with active caspases (an indicator of apoptosis), CLNC were stained with Apostat (pan-caspase inhibitor) (1:100) (R&D, Minneapolis, MN, USA) for 30 min at 37°C in a 5% CO_2 incubator, washed in PBS and analyzed on the flow cytometer.

Proliferation assay

After 24 h of treatment with ConA, control CLNC or siRNA NUPR1-transfected CLNC were treated with MTT solution (0.5 mg/ml) for 2 h at 37°C in a 5% CO_2 incubator. In the presence of oxygen derived from mitochondria MTT is reduced to dark formazan crystals, serving as an indicator of cell respiration and indirectly, of cell number and proliferation [12]. The formazan crystals were then dissolved in DMSO. The absorbance was measured in an automated microplate reader (LKB 5060-006, LKB Instruments, Vienna, Austria) at 540 nm and the background at 670 nm was subtracted. The proliferation index was calculated as the ratio between proliferation of ConA-stimulated cells and control cells.

Immunoblot

CLNC (10^6) were disrupted in lysis buffer containing 62.5 mM Tris-HCl (pH 6.8, 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue, and were subjected to electrophoresis on a 15% SDS-polyacrylamide gel. All samples were electrotransferred to polyvinylidene difluoride membranes at 5 mA/cm^2, using a semi-dry blotting system (Fastblot B43, Biorad, Munich, Germany). The blots were blocked with 5% w/v bovine serum albumin (BSA) in PBST buffer (80 mM Na_2HPO_4; 20 mM NaH_2PO_4; 100 mM NaCl; 0.1% Tween-20) and probed with specific rabbit antibodies raised against β-actin (1:500), tubulin (1:500) (Abcam, Cambridge, UK) and NUPR1 (1:500) (SantaCruz Biotechnology) in 1% blocking buffer, followed by incubation with secondary antibody donkey anti-rabbit HRP at 1:10000 (GE Healthcare, Buckinghamshire, England) in 1% blocking buffer. Detection was performed by the substrate for horseradish peroxidase (HRP), Luminata Crescendo (Milipore Corporation, Billerica, AM, USA); photographs were made using X-ray films (Kodak, USA). Protein production was calculated by Scion Image Alpha 4.0.3.2 (Scion Corporation, Frederick, MD, USA) and was expressed relative to the production of β-actin or tubulin.

RNA isolation, reverse transcription and Real-time PCR

CLNC were lysed using RNAzol (Metabion, Martinsried, Germany) and RNA was obtained; reverse transcription to cDNA and real-time PCR was performed as previously described [13]. The primer pairs for β-actin were 5'-GACCTGACAGACTACC-3 and 5'-GGCATAGAGGTCTTTACGG-3' (NM_007393.2); NUPR1 primer pairs were purchased as Quantitect Primer Assays from Qiagen (Germantown, MD, USA). NUPR1 mRNA expression was calculated according to the formula $2^{-(Ct-Cta)}$, where C_t is the cycle threshold of the gene of interest and C_{ta} is the cycle threshold value of the housekeeping gene (β-actin). Data were obtained from a real-time PCR apparatus and quantitatively analyzed using SDS 2.1 software (Applied Biosystems, Woolston, UK).

Statistical analysis

Statistical analysis was performed using the Student's t test (Statistica 6.0; StatSoft,Inc., Tulsa, OK, USA). The results were presented as means±standard deviation (SD); $p<0.05$ were considered statistically significant.

RESULTS

The relation of NUPR1 and mitogen-triggered lymphocyte proliferation

As it has been documented that NUPR1 can be involved in cell proliferation [8], we investigated whether NUPR1 expression correlates with lymphocyte proliferation. The expansion of lymphocytes was initiated with concanavalin A (ConA), a non-specific T cell mitogen that binds and crosslinks components of the T cell receptor, thereby activating signaling mechanisms for cell division. ConA indeed stimulated proliferation as judged by the MTT assay (0.253±0.086 in control cell cultures vs. 0.510±0.079 in ConA-treated, p=0.019). However, when these cells were exposed to ConA for 24 h in optimal culture conditions, the expression of NUPR1 mRNA subsided (Fig. 1A). This phenomenon was accompanied by lower NUPR1 protein expression (Fig. 1B) after 24 h cultivation with mitogen, suggesting that NUPR1 expression and proliferation are inversely correlated. However, attenuation of *NUPR1* mRNA expression with specific siRNA resulted in impaired ConA-stimulated CLNC proliferation (Fig. 1C). This implies that the activity of NUPR1 is probably necessary, at least for the initiation of lymphocyte proliferation. Notably, the efficiency of *NUPR1* mRNA silencing was 100% (Fig. 1D).

NUPR1 expression correlates with stress-induced apoptosis

Interestingly, lymphocytes isolated from cervical lymph nodes and immediately tested for the presence of apoptotic cells have a small rate of 8% of apoptosis (Fig. 2A). This coincides with a relatively high expression of *NUPR1* mRNA (Fig. 2B), probably due to stressful events during the cell isolation procedure. However, when these cells are placed in a culture plate with optimal conditions for their survival, the apoptosis rate increases over time and reaches a steady state after 24 h (Fig. 2A). Simultaneously, we tested *NUPR1* mRNA expression and found that its expression transiently increases up to 6 h and then subsides after 24 h (Fig. 2B). Seemingly, the *NUPR1* expression precedes apoptosis in lymphocytes. Also, the presence of the NUPR1 protein (Fig. 2C) correlates well with the expression of mRNA. What is more, lymphocyte apoptosis induced by staurosporine (an antibiotic that unselectively inhibits protein kinases by blocking the ATP-binding domain; Fig. 2D) leads to an increase in NUPR1 mRNA and protein expression (Fig. 2 E, F), suggesting a positive correlation between NUPR1 action and apoptosis induction.

NUPR1 does not promote apoptosis in T lymphocytes

In order to confirm the suggested role of NUPR1 in apoptosis induction in lymphocytes, NUPR1 mRNA was blocked using siRNA. When NUPR1 was inhibited, staurosporine was not as efficient in inducing apoptosis as when NUPR1 was active (Fig. 3A). However, when we isolated $CD3^+$ cells, staurosporine provoked apoptosis of these cells, as in the control cultures (Fig. 3B), suggesting that NUPR1 is not mandatory for apoptosis induction in T lymphocytes.

DISCUSSION

Our study has revealed that NUPR1 could have a role in the initiation of lymphocyte proliferation stimulated by mitogen as well as in apoptosis induction of non-T cells in the lymph nodes of C57BL/6 mice. NUPR1 expression is usually high in tumor cells and it is thought that it stimulates their proliferation and drug resistance. However, it might have an opposite role in normal, non-transformed somatic cells since its deletion enables the rapid proliferation of pancreatic beta cells [8]. In this study, two opposing results were obtained: attenuation of NUPR1 impaired lymphocyte proliferation, but also, NUPR1 expression was significantly lower 24 h after the administration of mitogen. Seemingly, NUPR1 might be mandatory for the initiation of proliferation, but it is not needed for subsequent steps of cell division and therefore its expression subsides. The observed lower NUPR1 expression after ConA stimulation cannot be an artifact since the non-treated mixed lymphocyte population in

the culture increase their NUPR1 expression compared to the baseline level detected immediately after cell isolation from the lymph nodes. This upregulation of NUPR1 is probably related to the initiation of cell apoptosis due to cell manipulations during the isolation procedure. It is remarkable that CLNC kept in the optimal conditions for their survival, exhibit around 30% of apoptosis after 24 h in culture. In addition, apoptosis induction by staurosporine also stimulates NUPR1 expression. This is, however, in conflict with data obtained from the cervical cancer cell line (HeLa cells), where NUPR1 binds the anti-apoptotic protein prothymosin α and blocks staurosporine-induced apoptosis [2]. The observed discrepancy in the relation between induced apoptosis and NUPR1 expression could be in the nature of the investigated cells, meaning that immortalized cells have a different physiology and thus NUPR1 might have a different function. The final proof that NUPR1 is actually related to CLNC apoptosis induction was obtained from the experiments where NUPR1 expression was inhibited by specific siRNA that interfered with translation of the NUPR1 protein. The observed reduction in cell apoptosis in the absence of NUPR1 clearly suggests that NUPR1 is mandatory for the initiation of apoptosis by staurosporine. However, $CD3^+$ cells were not protected from apoptosis induction when NUPR1 was absent. Although our results do not imply an anti-apoptotic role of NUPR1, Weis et al. [11] suggested that NUPR1 functions as a blocker of $CD3^+$ lymphocyte apoptosis since in both physiological conditions and after induction of inflammation, NUPR1 deletion coincides with increased $CD3^+$ apoptosis. Due to experimental restrictions we were not able to identify the possible immune cell population in the lymph node that requires NUPR1 expression for initiation of the apoptotic process, but it can be speculated that these might be B lymphocytes that represent the second predominant population in cervical lymph nodes (around 30%). This percentage actually coincided with the usual number of B cells in cervical lymph nodes in healthy animals (our unpublished results). Indeed, it was shown that B cells can succumb to staurosporine-induced apoptosis *in vitro* [14]. Staurosporine is a broad-spectrum protein kinase inhibitor and it initiates apoptosis in both normal and transformed cells [15,16]. Although we cannot ignore the fact that NUPR1 is important for the execution of apoptosis in other immune cell populations present in lymph nodes, such as dendritic cells (1.5-2.5%), macrophages (3-4%) and NK cells (1-2.5%), these cells are relatively low in number, and their participation in the obtained amount of apoptotic cells is probably negligible.

Finally, it can be concluded that NUPR1 is important for initiation of the proliferation of T cells and the initiation and propagation of apoptosis in immune cells other than T lymphocytes.

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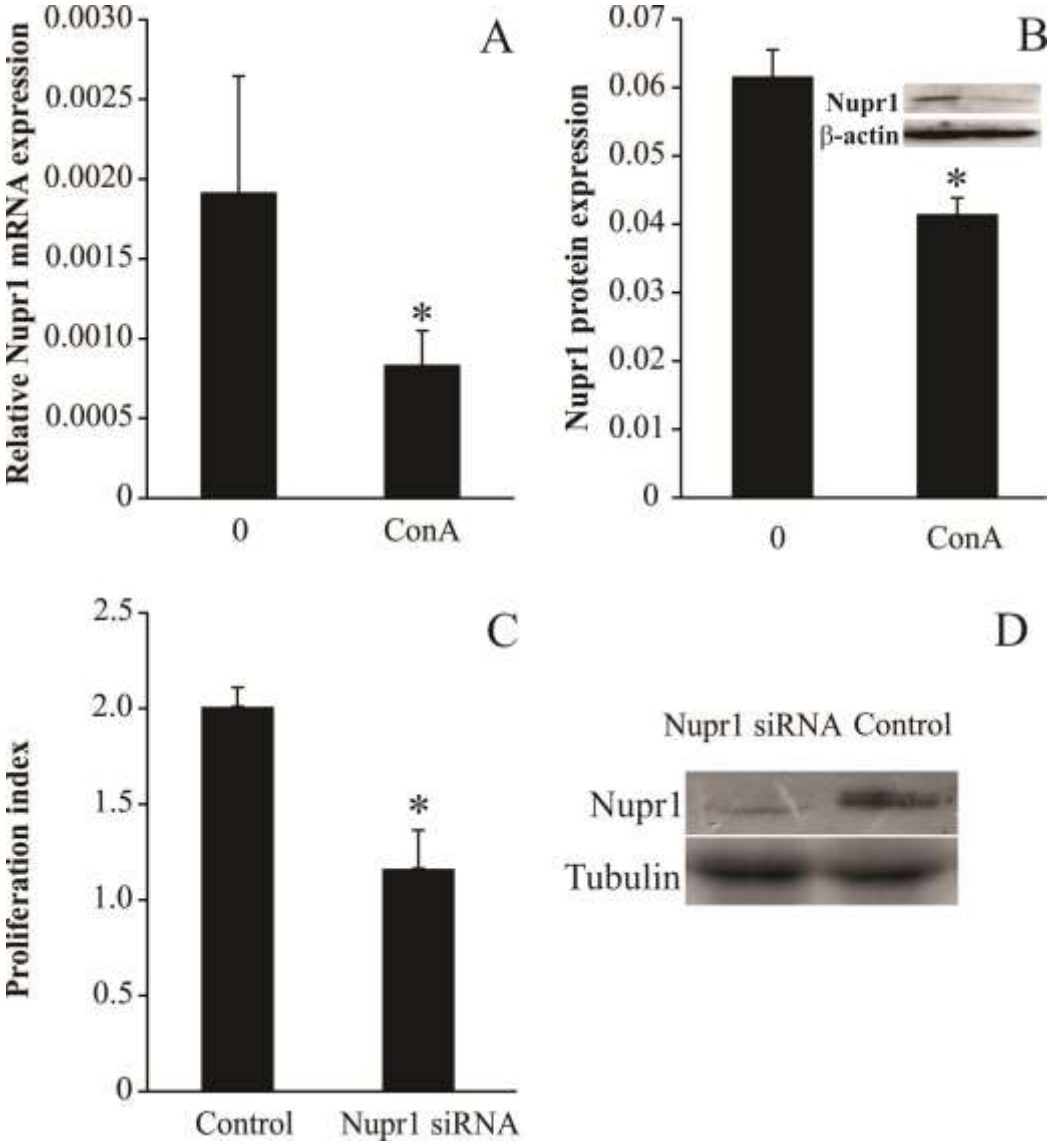
Figure Legends

Fig. 1. The role of NUPR1 in T cell proliferation. Cervical lymph node cells were incubated for 24 h with ConA. **A** – NUPR1 mRNA expression estimated by real-time PCR. **B** – NUPR1 protein expression estimated by immunobloting. The results are the means from three experiments. NUPR1 expression was attenuated by siRNA NUPR1 and cells were treated with ConA for 24 h prior to the MTT assay. **C** – Results of cell proliferation displayed as the proliferation index (the ratio of ConA-treated cells and control cells). **D** – the efficiency of NUPR1 silencing. * denotes $p<0.05$ between ConA-treated and untreated cells (0).

Fig. 2. The role of NUPR1 in immune cell apoptosis. Cervical lymph node cells were either tested immediately after isolation (0 h) or kept in RPMI-1640 and 5% FBS medium in the incubator for 6 h or 24 h. **A** – Apoptosis detected by the determination of caspase activity (flow cytometry). **B** – NUPR1 mRNA expression determined by real-time PCR. **C** – NUPR1 protein expression estimated by immunoblotting. A representative blot is displayed adjacent to the graph. **D** – Cervical lymph node cells treated with staurosporine (1 μM); apoptosis was determined by annexin-FITC staining immediately after isolation (0 h) or 3 h after the treatment. **E** – NUPR1 mRNA expression and **F** – NUPR1 protein expression 1 h and 3 h after staurosporine administration. The results of one representative experiment out of three with similar results are displayed. * denotes $p<0.05$ between values detected in cells at time points 6 h and 24 h vs. 0 h (A, B, C), or 3 h vs, 0 h (D, E, F); # denotes $p<0.05$ between values detected in staurosporine-treated vs. untreated cells.

Fig. 3. NUPR1 is not mandatory for apoptosis in $CD3^+$ lymphocytes. Cervical lymph node cells were treated with control or NUPR1 siRNA and apoptosis was induced with staurosporine (1 μM). After 3 h of incubation cells with active caspases were detected by flow cytometry (Apostat) (**A**). $CD3^+$ cells were isolated from cervical lymph node cells, treated with control or NUPR1 siRNA, and then incubated for 3 h with staurosporine (1 μM). Apoptosis was detected by measuring the percentage of cells with active caspases by flow cytometry (Apostat) (**B**). Results are displayed as the fold increase compared to the values measured in untreated cells (0) and are the means from three experiments. * denotes $p<0.05$ between values detected in staurosporine-treated cells vs. untreated cells.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Relative Nupr1 mRNA expression
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Nupr1 protein expression
0.07
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0.05
0.04
0.03
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Nupr1
β-actin
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ConA
C
Proliferation index
2.5
2.0
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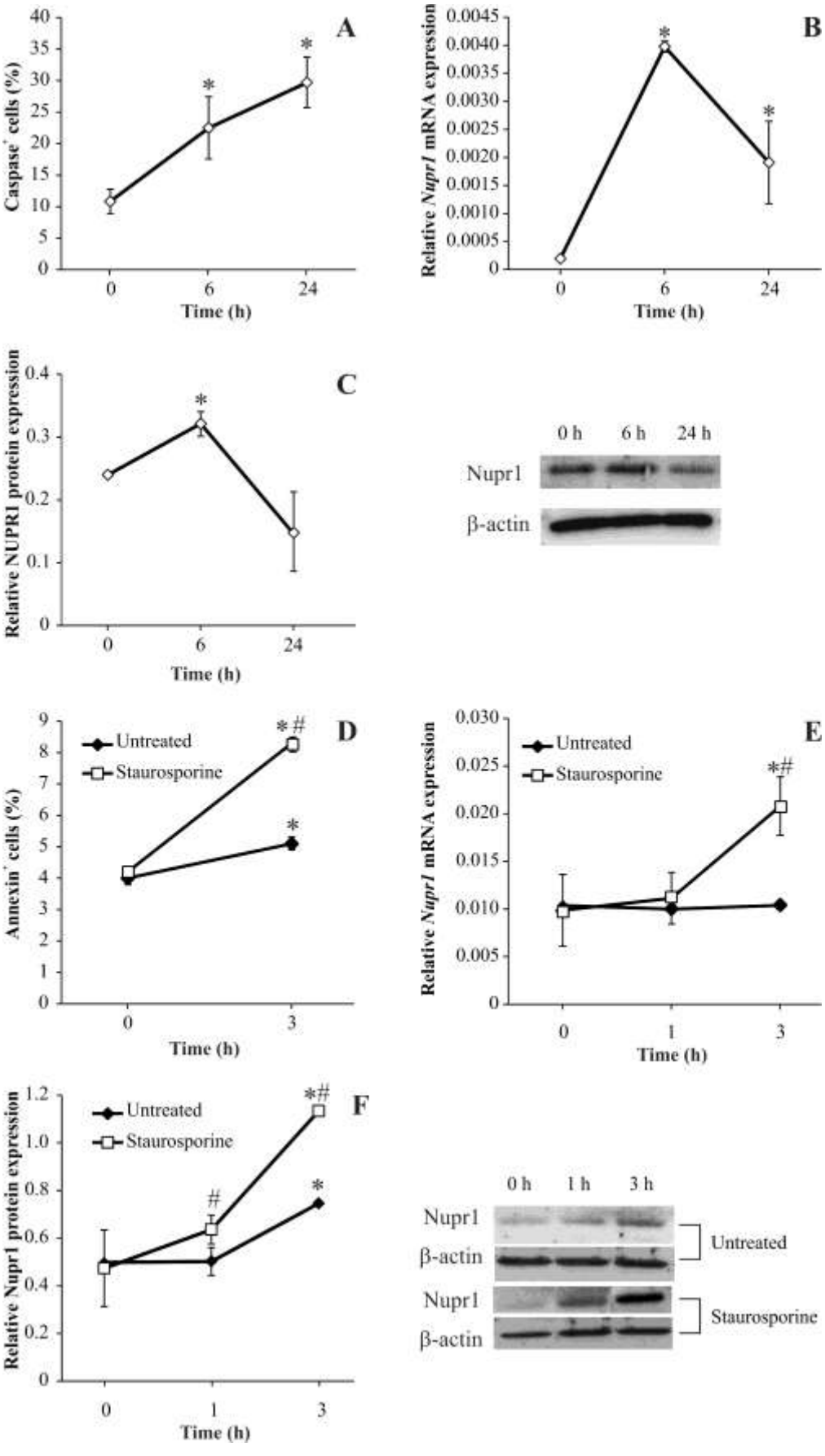
Fig. 1.


A
Caspase+ cells (%)
Time (h)
B
Relative Nupr1 mRNA expression
Time (h)
C
Relative NUPR1 protein expression
Time (h)
0 h
6 h
24 h
Nupr1
β-actin
D
Untreated
Staurosporine
Annexin+ cells (%)
Time (h)
E
Untreated
Staurosporine
Relative Nupr1 mRNA expression
Time (h)
F
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Time (h)
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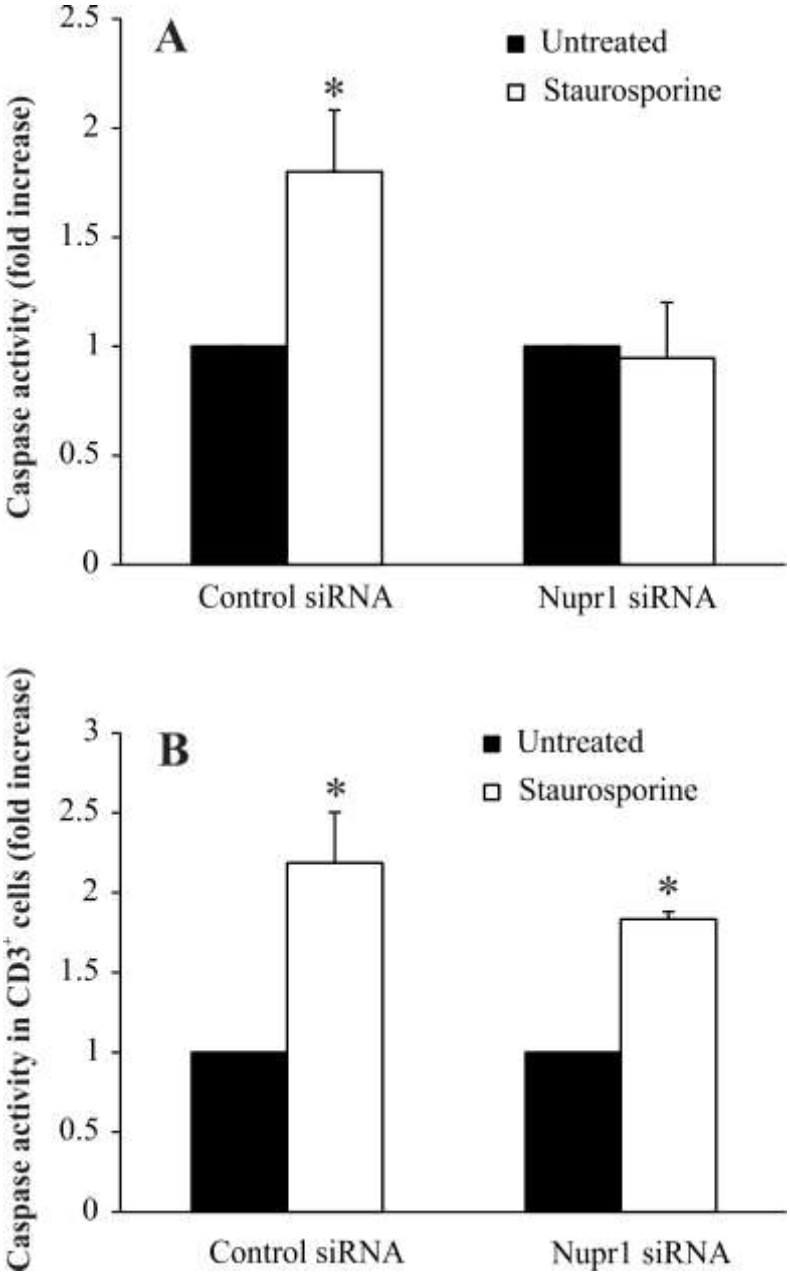
β-actin
Untreated
Staurosporine

Fig. 2.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Untreated
Staurosporine
Caspase activity (fold increase)
2.5
2
1.5
1
0.5
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Control siRNA
Nupr1 siRNA
B
Untreated
Staurosporine
Caspase activity in CD3+ cells (fold increase)
3
2.5
2
1.5
1
0.5
0
*
*
Control siRNA
Nupr1 siRNA

Fig. 3.