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Research Article

Taxol activates inducible nitric oxide synthase in rat astrocytes: the role of MAP kinases and NF-κB

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Abstract. Taxol is a microtubule-stabilizing agent that has recently been shown effective in the treatment of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. As astrocytes could modulate central nervous system (CNS) autoimmunity through inducible nitric oxide synthase (iNOS)-mediated production of immunoregulatory free radical nitric oxide (NO), we investigated the effect of taxol on NO synthesis in rat astrocytes. Taxol, either alone or in combination with interferon-γ, induced NO generation in primary astrocytes and astrocytoma C6 cells in a dose- and time-dependent manner. Accordingly, the drug markedly up-regulated the expression of both iNOS mRNA and protein in astrocytes. The observed effect of taxol was mediated through induction of iNOS transcription factors NF-κB and IRF-1, and required the activation of p38 MAP kinase and JNK. Finally, NO release by taxol-stimulated astrocytes was blocked with the microtubule-depolymerizing agent colchicine, suggesting the involvement of a microtubule-stabilizing activity of taxol in the observed effect.

Key words. Taxol; nitric oxide; iNOS; astrocytes; MAP kinase; NF-κB.

Taxol is a plant-derived antineoplastic drug which benefits patients with advanced breast, ovary and non-small-cell lung carcinoma [1]. The therapeutic effect of taxol is based on its ability to block G2/M cell cycle progression by stabilizing microtubules against depolymerization, therefore preventing mitosis and promoting apoptosis of tumor cells [2–4]. Taxol has also been shown capable of exerting lipopolysaccharide (LPS)-mimetic actions, including induction of nitric oxide (NO) and tumor necrosis factor (TNF) production in macrophages, activities which are partly responsible for its tumoricidal properties [5–7]. The ability of taxol to influence expression of LPS-inducible genes suggested that it could also modulate inflammation. Indeed, taxol exerted potent anti-inflammatory effects resulting in attenuation of the disease symptoms in animal models of rheumatoid arthritis and multiple sclerosis [8, 9].

Free radical molecule NO, produced by inducible NO synthase (iNOS)-mediated intracellular oxidation of L-arginine, has been implicated in both the effector and regulatory arms of immunity [10]. There is a growing body of evidence that NO could contribute to immune-mediated tissue destruction, but might also exert protective immunomodulatory functions in inflammatory autoimmune diseases [11, 12]. In inflammatory disorders of the central nervous system (CNS), such as multiple sclerosis and its animal model experimental autoimmune encephalomyelitis (EAE), elevated NO production from glial cells and infiltrating macrophages participates in disease symptoms by causing demyelination and subse-

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quent axonal loss [13, 14]. On the other hand, a number of recent studies indicate that iNOS-mediated NO synthesis by peripheral macrophages and glial cells could also play a vital role in attenuating the disease progress through inhibition of the activity of encephalitogenic T cells [15].

Although taxol has proved effective in up-regulating macrophage NO release and suppressing EAE [6, 7, 9], its influence on astrocyte NO production has not been analyzed to date. We therefore investigated the effect of taxol on iNOS activation and subsequent NO production in rat primary astrocytes and the C6 astrocytoma cell line.

Materials and methods

Reagents

Fetal calf serum (FCS), RPMI-1640 and phosphate-buffered saline (PBS) were purchased from ICN (Costa Mesa, Calif.). Recombinant rat interferon-γ (IFN-γ) was obtained from Holland Biotechnology (Leiden, The Netherlands). Taxol (paclitaxel), sulfanilamide, naphthylenediamine dihydrochloride, actinomycin D, cycloheximide, MG132, SB203580, L-leucine-methyl-ester, DMSO and rabbit anti-rat iNOS antibody were all from Sigma (St Louis, Mo.). Moloney leukemia virus reverse transcriptase and Taq polymerase were obtained from Eurogentec (Seraing, Belgium). RNA Isolator was purchased from Genosys (Woodlands, Tex.), while random primers were from Pharmacia (Uppsala, Sweden). Rabbit anti-rat phospho-extracellular signal-regulated kinase (ERK) and c-Fos, mouse anti-rat phospho-p38 mitogen-activated protein kinase (MAPK), phospho-JNK and phospho-IκB, goat anti-rat phospho-c-Jun and rabbit anti-rat IRF-1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.).

Cells and cell culture

Astrocytes were isolated from brains of newborn Dark Agouti rats maintained at the animal facility of the Institute for Biological Research (Belgrade, Serbia and Montenegro) as previously described [16]. The mixture of glial cells was kept in HEPES-buffered RPMI-1640 medium supplemented with 5% FCS, 2 mM glutamine, antibiotics, sodium pyruvate and 6 g/l glucose in 25-cm^2 tissue culture flasks at 37 °C in a humidified atmosphere with 5% CO_2. After 10 days of cultivation, cultures were mechanically shaken to dislodge oligodendrocytes and microglia, and astrocytes were further purified by repetition of trypsinization (0.25% trypsin and 0.02% EDTA) and replating. The cells used in experiments were obtained after the third to fourth passage when they were more than 97% positive for glial fibrillary acidic protein and less than 1% positive for the microglial surface molecule CD11b, as determined by flow cytometry analysis with appropriate FITC-labeled antibodies (data not shown). The remaining 2% of cells were probably immature astrocytes or astrocytes that had lost their GFAP expression, as previously reported [17]. Rat astrocytoma cell line C6 was purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). Astrocytes and C6 cells were incubated with different agents in 200 µl of culture medium.

Nitrite measurement

Nitrite accumulation, an indicator of NO production, was assayed by the Griess reaction [18]. Cell culture supernatant (50 µl) was added to an equal volume of Griess reagent (a mixture at 1:1 of 0.1% naphthylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4). After 10 min at room temperature, the absorbance was measured at 570 nm in a microplate reader. The nitrite concentration was calculated from an $NaNO_2$ standard curve.

RT-PCR determination of iNOS and IRF-1 mRNA

Total RNA from C6 cell cultures incubated for 6 h with taxol was isolated with RNA Isolator, according to the manufacturer's instructions. The RNA (1 µg) was reverse transcribed using Moloney leukemia virus reverse transcriptase and random primers. PCR amplification of cDNA with primers specific for iNOS/IRF-1 and GAPDH as a housekeeping gene was carried out in the same tube in a Thermojet (Eurogentec, Seraing, Belgium) thermal cycler as follows: 30 s of denaturation at 95 °C, 30 s of annealing at 51 °C for iNOS or 58 °C for IRF-1, and 30 s of extension at 72 °C. The number of cycles (25 for GAPDH and 30 for iNOS and IRF-1), ensuring non-saturating PCR conditions, was established in preliminary experiments. For iNOS, the sense primer was 5′-AGAGAGATCCGGTTCACA-3′, and the antisense primer was 5′-CACAGAACTGAGGGTACA-3′, corresponding to positions 88–105 and 446–463, respectively, of the published rat iNOS mRNA sequence (GenBank accession number S71597); the PCR product was 376 bp long. For IRF-1, the primers were: sense, 5′-GACCAGAGCAGGAACAAG-3′; antisense, 5′-TAACTTCCCTTCCTCATCC-3′, corresponding to positions 483–500 and 881–899, respectively, of the published rat IRF-1 mRNA sequence (M34253); the PCR product was 417 bp long. The primers for GAPDH were: sense, 5′-GAAGGGTGGGGCCAAAAG-3′; antisense, 5′-GGATGCAGGGATG ATGTTCT-3′, corresponding to positions 371–388 and 646–665 of the published rat GAPDH mRNA sequence (AB017801); the PCR product was 295 bp long. PCR products were visualized by electrophoresis through an agarose gel stained with ethidium bromide. Gels were photographed and results were analyzed by densitometry.

Cell-based ELISA

The expression of p-p38 MAPK, p-ERK, p-IκB, c-Fos, p-c-Jun, p-JNK, iNOS and IRF-1 in taxol-stimulated C6 cells was determined using the original protocol for the cell-based ELISA [19]. The cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and washed three times with PBS containing 0.1% Triton X-100 (PBS/T). Endogenous peroxidase was quenched with 0.6% H_2O_2 in PBS/T for 20 min, and cells were washed three times in PBS/T. Following blocking with 10% FCS in PBS/T for 1 h at 37 °C, cells were incubated for 1 h with the primary antibody in PBS/T containing 1% bovine serum albumin (BSA) at 37 °C. After washing the cells four times with PBS/T for 5 min, they were incubated for 1 h with the second antibody (HRP) in PBS/T containing 1% BSA at 37 °C. Subsequently, cells were washed and incubated with 50 µl of a solution containing 0.4 mg/ml OPD, 11.8 mg/ml Na_2HPO_4. $2H_2O$, 7.3 mg/ml citric acid and 0.015% H_2O_2 for 30 min at room temperature in the dark. The reaction was stopped with 25 µl of 3 M HCl, and the absorbance at 492 nm was determined in a microplate reader.

Statistical analysis

The values presented are means ± SD of triplicate observations from a representative of at least three experiments with similar results, unless indicated otherwise. To analyze the significance of the differences between various treatments, we used analysis of variance (ANOVA), followed by the Student-Newman Keuls test. A p value less than 0.01 was considered to be significant.

Results

Taxol stimulates NO production in primary astrocytes and C6 cells

In comparison with unstimulated cultures, treatment with taxol for 48 h caused a significant dose-dependent increase in nitrite accumulation, an indicator of NO production, in supernatants of primary rat astrocyte cultures (fig. 1A). The additive effect on astrocyte NO production was observed if taxol was combined with IFN-γ (fig. 1A), a proinflammatory cytokine able to induce NO release [10] (fig. 1A). No difference in the ability of taxol to induce NO production was observed if astrocyte cultures were pretreated with 5 mM of the selective microglial toxin L-leucine-methyl-ester (5.5 ± 0.4 µM vs 5.7 ± 0.2 µM of nitrite in taxol-treated cultures without or with L-leucine-methyl-ester, respectively; $p > 0.05$), which has been routinely used to obtain microglia-free astrocyte cultures [17, 20]. This indicated that a very low residual number of contaminating microglia (approx. 1%) did not significantly contribute to taxol-triggered NO generation in primary astrocytes. A similar dose-dependent increase in NO production was also evident in the taxol-treated C6 rat astrocytoma cell line (not shown), further confirming such an assumption. In the time dependence experiments, taxol-mediated NO synthesis in C6 cells showed an almost linear rise through 72 h of incubation (fig. 1B). The difference in NO production by taxol-treated astrocytes or C6 cells at 48 h (fig. 1A, B) was not consistently observed, and was probably due to experiment-to-experiment variations. As similar results were obtained with primary astrocytes and astrocytoma cells, further experiments were therefore performed interchangeably on either cell type.

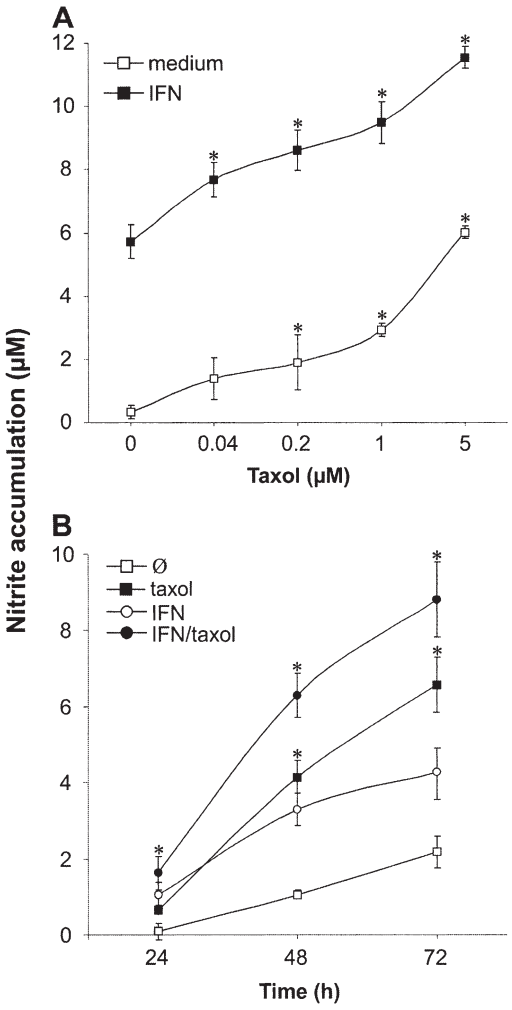

Figure 1. Taxol stimulates NO production in primary astrocytes and C6 cells. Primary rat astrocytes (*A*) and C6 cells (*B*) were incubated with or without IFN-γ (500 U/ml), in the presence or absence of various concentrations (*A*) or 5 µM (*B*) of taxol. The cell culture supernatants were collected for NO determination after 48 h (*A*) or at the indicated time points (*B*). *p < 0.01 compared to cultures without taxol.

Taxol up-regulates iNOS and IRF-1 expression in astrocytes and C6 cells

A fairly specific iNOS inhibitor aminoguanidine [21] completely prevented NO production in taxol-stimulated C6 cells (7.2 ± 0.5 and 1.1 ± 0.2 µM for control and aminoguanidine-treated cultures, respectively; $p < 0.01$), indicating that the observed effect of taxol was iNOS dependent. To assess whether taxol could affect iNOS enzymatic activity, C6 cells were pretreated with IFN-γ for 24 h to induce iNOS expression, and any further induction was blocked by inhibiting transcription or translation with actinomycin D or cycloheximide, respectively. The ability of taxol to stimulate NO production in these conditions was completely blocked (fig. 2A), indicating that taxol influenced iNOS induction rather than its catalytic activity. Such an assumption was further substantiated by the ability of taxol to induce the expression of iNOS protein in C6 cell cultures, which coincided with the up-regulation of the important iNOS transcription factor IRF-1 (fig. 2B). Similar effects were observed in rat primary astrocytes (data not shown). The induction of iNOS and IRF-1 by taxol was confirmed at the RNA level, as treatment with taxol markedly up-regulated the expression of both iNOS and IRF-1 transcripts in primary astrocytes (fig. 2C).

The effect of taxol on NF-κB and MAPK activation in C6 astrocytoma cells

In murine macrophages, taxol has been shown to mimic the ability of LPS to activate iNOS transcription factor NF-κB and all three MAPK family members – p38 MAPK, ERK and JNK [22, 23]. We therefore next examined the influence of taxol on these signal transduction pathways, as well as on MAPK-dependent c-Jun activation and c-Fos expression in C6 cells. As MAPKs and c-Jun are activated by phosphorylation, and the activation of NF-κB follows the phosphorylation of its inhibitor IκB, we measured the concentration of the phosphorylated forms of these molecules. A significant up-regulation of phosphorylated IκB was clearly observed within 30 min of incubation with taxol, reaching a maximum after 60 min and returning to the basal level after an additional 1 h (fig. 3A). Similarly, phosphorylation of p38 MAPK was transiently increased 30 min after the treatment with taxol (fig. 3B), while the activation of JNK and its substrate c-Jun was readily augmented 15 min from application of taxol and remained elevated throughout the time course examined (fig. 3C, D). On the other hand, although ERK phosphorylation and c-Fos expression were readily induced in C6 cells by treatment with serum, taxol failed to a exert similar effect at any of the time points tested (fig. 3E, F).

NF-κB and p38 MAPK mediate taxol induction of astrocyte iNOS and IRF-1

Since taxol up-regulated the activation of NF-κB and p38 MAPK in C6 cells (fig. 3), we next sought to examine the involvement of these molecules in taxol-mediated induc-

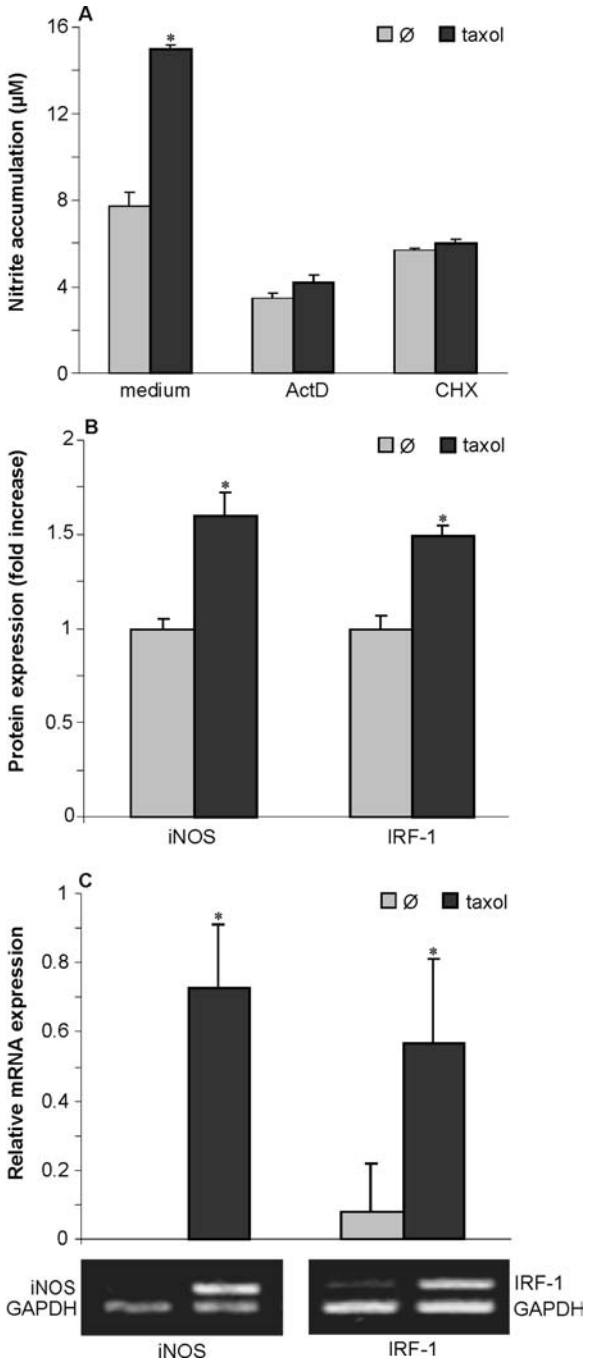

Figure 2. Taxol up-regulates iNOS and IRF-1 expression in astrocytes and C6 cells. (*A*) C6 cells were pretreated with IFN-γ (500 U/ml) for 24 h, extensively washed, and incubated for an additional 24 h with or without actinomycin D (ActD; 5 µg/ml) or cycloheximide (CHX; 5 µg/ml), in the presence or absence of taxol (5 µM). (B) C6 cells were incubated alone or with taxol (5 µM) for 36 h and cELISA for iNOS and IRF-1 was performed. The data are presented as fold increase (A^{492}) relative to the control cultures without taxol. (*C*) Primary astrocytes were incubated alone or with taxol (5 µM) for 6 h and total RNA was isolated for measurement of iNOS and IRF-1 mRNA by RT-PCR. The values representing the ratio between iNOS or IRF-1 and GAPDH signals are presented as means ± SD from three separate experiments. Gel photographs from the representative experiment are presented. *$p < 0.01$ compared to cultures without taxol.

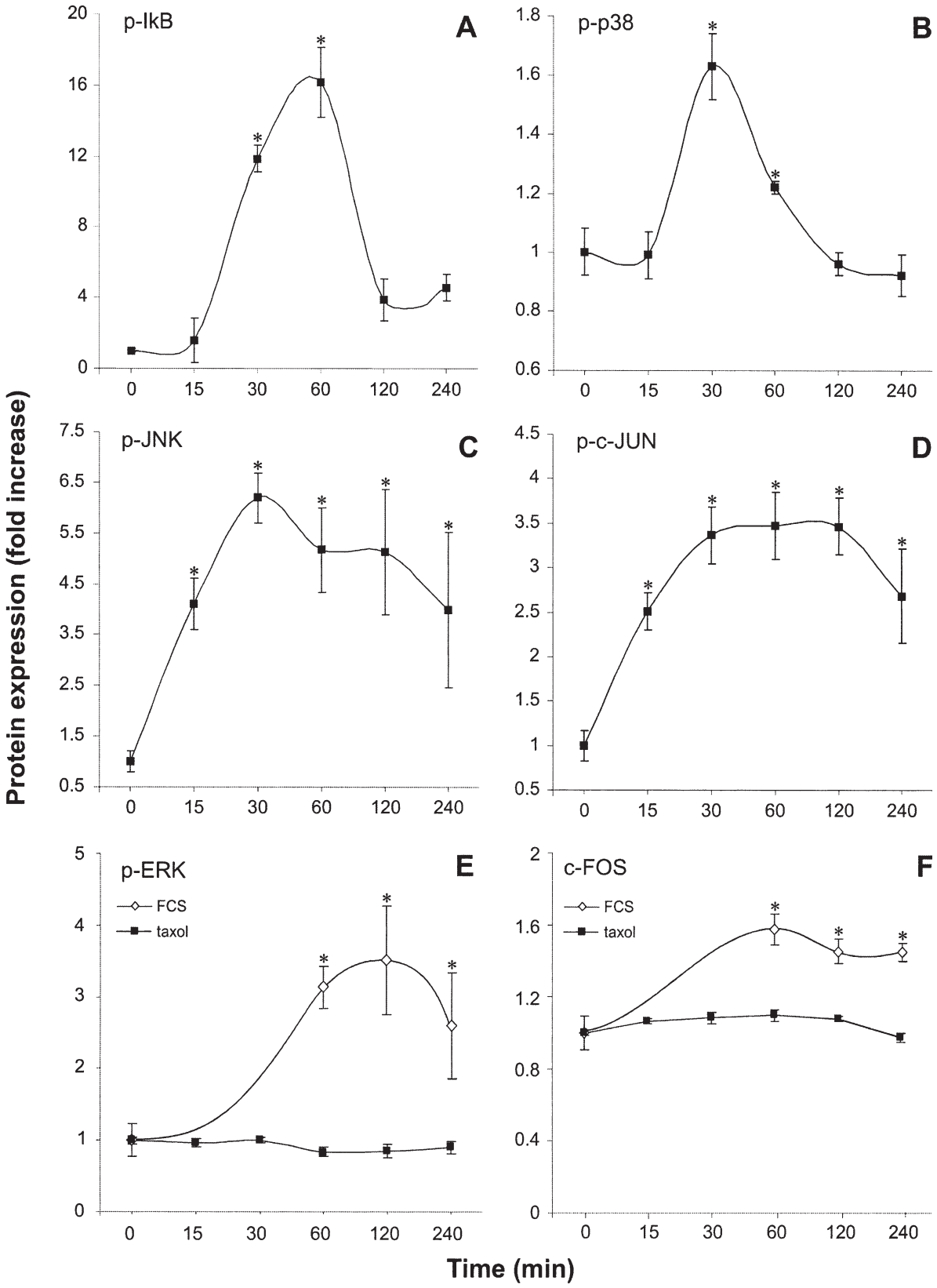

Figure 3. The effect of taxol on NF-κB and MAPK activation in C6 cells. For cELISA measurement of p-IκB (*A*), p-p38 (*B*), p-JNK (*C*), p-c-Jun (*D*), p-ERK (*E*) and c-Fos (*F*), serum-starved (0.5% FCS overnight) C6 cells were incubated for different time periods with 5 μM of taxol (A–F) or 10% FCS (*E*, *F*). The results are presented as fold increase (A^{492}) relative to values obtained in untreated cultures (0 min). * $p < 0.01$ compared to cultures without taxol.

tion of astrocyte iNOS. For this, we used SB230580, a selective inhibitor of p38 MAPK, and MG132, a proteasome inhibitor that blocks NF-κB activation. In accordance with previous observations, treatment with either SB230580 or MG132 markedly impaired the ability of taxol to stimulate NO production in C6 cells (fig. 4A). Accordingly, both inhibitors prevented taxol-induced upregulation of iNOS mRNA in primary astrocytes (fig. 4B). Interestingly, the interference with NF-κB or p38 MAPK activation also blocked taxol-mediated expression of IRF-1 in primary astrocytes, indicating that the induction of this iNOS transcription factor by the drug might depend on both NF-κB and p38 MAPK activity. Similar results were obtained with another NF-κB inhibitor, an antioxidant agent pyrrolidine dithiocarbamate (not shown), thus minimizing the possibility that MG132 action was mediated by some NF-κB-independent consequence of proteasome inhibition.

Colchicine inhibits taxol-induced NO production in C6 astrocytoma cells

Finally, to determine if taxol stimulated NO production in astrocytes through its effect on microtubule dynamics, we used the microtubule-depolymerizing agent colchicine. Pretreatment with colchicine significantly reduced taxol-induced NO production in C6 cells (fig. 5), without affecting their viability (not shown). This result indicates that taxol could indeed trigger iNOS induction in astrocytes through mechanisms that, at least in part, involve microtubule stabilization.

Discussion

In the present study we show that the microtubule-stabilizing agent taxol can stimulate iNOS expression and subsequent NO production in both primary astrocytes and the C6 astrocytoma cell line, through mechanisms probably involving the activation of the MAPK signaling pathway and iNOS transcription factors NF-κB and IRF-1. While taxol has been previously reported to induce TNF, interleukin-1 and iNOS expression in macrophages [5–7, 24, 25], this is the first study on the ability of taxol to modulate the inflammatory properties of astrocytes, which represent the most abundant glial cell type in the CNS.

Simultaneous binding of NF-κB and IRF-1 to their consensus sequences in the iNOS promoter has been shown to be required for optimal iNOS transcription in macrophages [26], and both transcription factors have been implicated in iNOS induction in astrocytes [27–29]. While activation and subsequent nuclear translocation of constitutively expressed NF-κB is mediated by phosphorylation of its inhibitor IκB [30], IRF-1 is activated mainly at the transcriptional level [31]. Since we demonstrated a taxol-mediated increase in phospho-

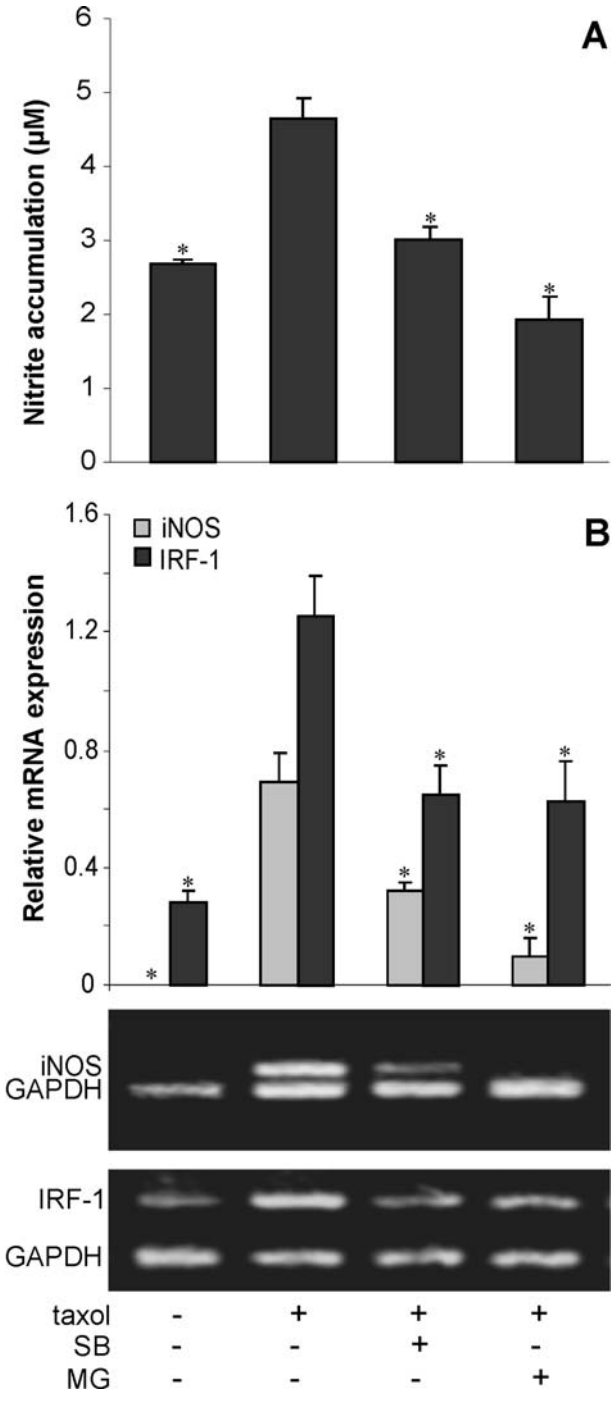

Figure 4. Taxol stimulates astrocyte iNOS and IRF-1 expression via NF-κB and p38 MAPK activation. (*A*) C6 cells were grown alone or in the presence of taxol (5 μM), in the presence or absence of p38 MAPK inhibitor SB230580 (SB; 5 μM) or the NF-κB inhibitor MG132 (MG; 1.2 μM). The amount of nitrite was measured after 48 h of incubation. (*B*) Rat primary astrocytes were treated as in *A*. After 6 h, total RNA was isolated and RT-PCR for iNOS and IRF-1 mRNA was carried out. The values representing the ratio between iNOS or IRF-1 and GAPDH signals are means ± SD from three separate experiments. Gel photographs from the representative experiment are presented. *$p < 0.01$ compared to cultures treated with taxol alone.

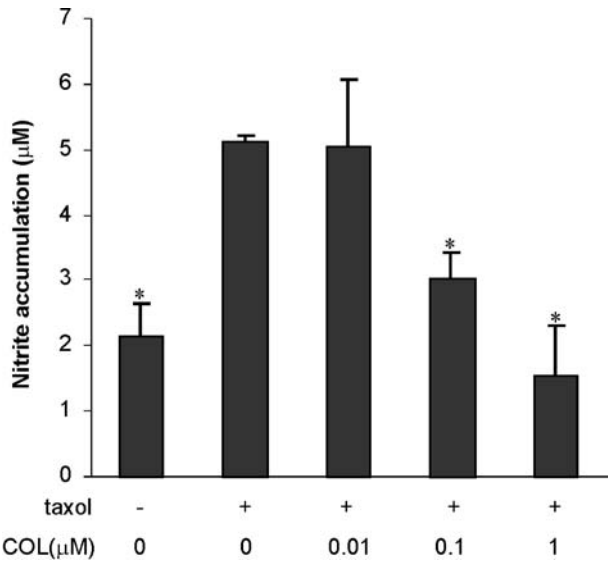

Figure 5. The effect of colchicine on taxol-induced NO production in C6 cells. Confluent C6 cells were incubated in medium or stimulated with taxol (5 μM) in the presence of various concentrations of colchicine (COL). Colchicine was added to cell cultures 15 min before taxol. The nitrite concentration was determined after 48 h of incubation. * $p < 0.01$ compared to cultures treated with taxol alone.

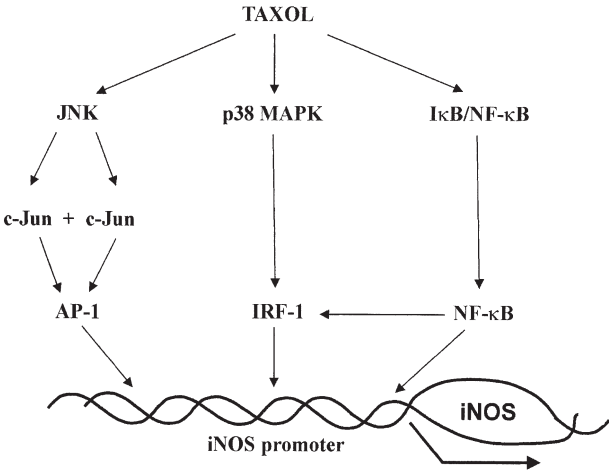

Figure 6. Hypothetical model of signaling pathways involved in taxol-induced iNOS activation in astrocytes.

IκB and IRF-1 mRNA concentration, co-ordinated activation of both transcription factors could be responsible for the induction of astrocyte iNOS by the drug. In addition, all three members of the MAPK signaling pathway (p38 MAPK, ERK and JNK) participate to different extents in iNOS induction by various stimuli in both rodent and human astrocytes [28, 32–35]. In the present study, taxol readily activated p38 MAPK and JNK in C6 astrocytoma cells, but failed to exert a similar effect on ERK. The latter observation is in contrast with the data obtained with murine macrophages, suggesting that ERK activation by taxol might be cell and/or species specific. MAPK signaling controls the activation of an important iNOS transcription factor, AP-1 [36], consisting of c-Jun and c-Fos proteins. In contrast to constitutively expressed c-Jun whose activation is initiated by p38 MAPK- or JNK-mediated phosphorylation, c-Fos is activated transcriptionally by ERK [37]. In accordance with its effects on MAPK activation in C6 cells, taxol increased JNK/p38 MAPK-controlled activation of c-Jun, while not affecting the ERK-dependent intracytoplasmic concentration of c-Fos. However, this might still lead to the AP-1 assembly that presumably contributed to taxol-triggered iNOS transcription in astrocytes, as functional AP-1 could also consist of phospho-c-Jun homodimers [38]. Based on the presented data, we propose a hypothetical model of the intracellular signaling responsible for astrocyte iNOS activation by taxol (fig. 6).

While taxol-mediated activation of NF-κB and MAPK signaling pathway has been reported in murine macrophages [4, 22, 23, 39, 40], this is the first study describing the ability of taxol to induce the expression of IRF-1 in both macrophages and astrocytes. This is not surprising, as IRF-1 is also efficiently induced by LPS [41, 42], and it has been postulated that taxol and LPS might share a common signaling machinery involving Toll-like receptor 4 [7, 23, 25, 39, 40, 43]. Interestingly, although IRF-1 induction has been usually associated with the activation of the JAK/STAT signaling axis [31], taxol-mediated induction of astrocyte IRF-1 in our study was apparently dependent on both NF-κB and p38 MAPK activation. This observation is consistent with the previously described involvement of NF-κB or p38 MAPK in IRF-1 induction by various stimuli in different cell types, including astrocytes [44–47]. However, we could not exclude the possibility that taxol might also activate the JAK/STAT pathway in our experiments. Precise mechanisms responsible for NF-κB/p38 MAPK-mediated activation of IRF-1 in taxol-stimulated astrocytes are currently under investigation in our laboratory.

Microtubules play an important role in LPS-triggered iNOS expression and subsequent NO synthesis in macrophages and vascular smooth muscle cells [48, 49]. Accordingly, taxol-mediated activation of macrophage NO release was partly exerted through stabilization of microtubules [48], which also seems to be one of the mechanisms involved in astrocyte iNOS induction by taxol in the present study. While colchicine, a microtubule-disrupting agent used in our experiments, inhibited the expression of iNOS mRNA in vascular smooth muscle cells [49], its iNOS-inhibitory action in macrophages was mediated exclusively through undefined post-transcriptional mechanism(s) [48]. In our hands, colchicine-mediated suppression of NO release by IFN-γ-stimulated fibroblasts coincided with the inhibition of the MAPK signaling pathway (unpublished observation). We are currently

investigating whether colchicine might employ similar mechanisms to block taxol-mediated induction of iNOS in astrocytes.

Beneficial immunosuppressive effects of taxol in the animal model of rheumatoid arthritis and EAE [8, 9] have been mostly attributed to the ability of the drug to directly block mitosis of autoreactive T cells. However, there is a question of the possible biological significance of taxol-triggered NO release by macrophages and astrocytes. Although NO has been considered as one of the major culprits responsible for demyelination and axonal loss in EAE and multiple sclerosis, it could also exert a protective action by down-regulating the function of antigen-presenting cells and inducing the apoptosis of myelin-reactive T cells [50–53]. While interference with the expansion of encephalitogenic T cells is primarily mediated by macrophage or dendritic cell-derived NO in lymphoid organs [51, 54], recent data point to resident glial cells as a potential source of NO responsible for in situ down-regulation of proinflammatory T cell activity in the CNS of animals with EAE [55–57]. The results of the present report might therefore be relevant for the protective action of taxol in EAE, as well as for human disease, since taxol has recently entered a phase II multicenter trial after promising effects in a phase I trial for secondary progressive multiple sclerosis [58]. Nevertheless, future studies are needed to confirm whether up-regulation of astrocyte-derived NO could contribute to the beneficial effect of taxol in CNS autoimmunity.

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