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Docetaxel inhibits cyclooxygenase-2 induction in vascular smooth muscle cells

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ABSTRACT

The aim of this study was to determine whether docetaxel affects expression of cyclooxygenase-2 (COX-2) in vascular smooth muscle cells. Cultured rat aortic smooth muscle cells (RASMCs) were stimulated with interleukin-1b (IL-1β). COX-2 expression level and ERK activity were evaluated by Western blot analysis. COX-2 expression as well as tubulin formation was also evaluated by immunocytochemical analysis. IL-1β induced COX-2 expression in RASMCs, which was inhibited by docetaxel (5–20 μg/ml) in a concentration-dependent manner. IL-1β increased ERK activity, which was not affected by docetaxel. IL-1β-induced COX-2 expression level was markedly augmented at 24 h after washing out docetaxel from the culture medium. Immunocytochemical analysis revealed that COX-2 immunoreactivity in RASMCs stimulated with IL-1β was decreased in the presence of docetaxel but was recovered at 24 h after washing out docetaxel, while docetaxel-induced change in tubulin formation, namely, polymerization of α-tubulin fibers, remained at 24 h after washing out docetaxel. The results suggest that docetaxel inhibits COX-2 induction, and this action of docetaxel is reversible and ERK-independent.

1. Introduction

Cyclooxygenase (COX) is a key enzyme catalyzing the rate-limiting step in synthesis of prostaglandin. While COX-1 is constitutively expressed in most tissues and has a general housekeeping function, COX-2 is normally undetectable but is inducible under circumstances such as inflammation and cancer. COX-2 is known to be over-expressed in tumor cells (Eberhart et al., 1994; Wolff et al., 1998), and high levels of prostaglandin E_2 (PGE_2) produced through the COX-2 pathway have been shown to be associated with increased proangiogenic factors, altered immune responses and enhanced metastatic potential (Young et al., 1987; Tsujii et al., 1997, 1998; Dohadwala et al., 2006; Sharma et al., 2003). Thus, COX-2 has been targeted for anticancer chemotherapy, and selective COX-2 inhibitors have recently been used in combination with other anticancer drugs for a synergic antitumor effect in colon and lung cancer models (Hida et al., 2002; Trifan et al., 2002). On the other hand, prostacyclin is known to have an anti-cancer effect through affecting growth, apoptosis, angiogenesis and metastasis of tumor cells (Cathcart et al., 2010). Prostacyclin synthase is down-regulated and PGE_2 synthase is up-regulated in tumor tissues compared with the levels in normal tissues (Stearman et al., 2005), and PGE_2/prostacyclin ratio is thought to play a role in invasion and metastasis of cancer cells (Dohadwala et al., 2006). The vascular wall is a major locus for prostacyclin production, and COX-2 is known to be induced by inflammatory stimulants, such as cytokines and lipopolysaccharide, in vascular smooth muscle cells (Rimarachin et al., 1994). Although the pathophysiological significance of prostacyclin released from the vascular wall in tumor invasion and metastasis remains to be clarified, it is reasonable to assume that prostacyclin from vascular cells as well as that from tumor cells have an anti-cancer role since vascular cells could be stimulated by cytokines during perivascular inflammation due to tumor invasion and metastasis, resulting in COX-2 induction and production of a large amount of prostacyclin in blood vessels.

Docetaxel, a derivative of paclitaxel (taxol), is widely used as a fundamental antineoplastic drug in the treatment of a variety of solid cancers such as breast cancer (Nabholtz et al., 1999), advanced gastric cancer (Van Cutsem et al., 2006), hormone-refractory prostate cancer (Petrylak et al., 2004), locally advanced squamous cell carcinoma of the head and neck (Vermorken et al., 2007) and advanced non-small cell lung cancer (Fossella et al., 2003). In tumor cells, taxanes such as docetaxel and paclitaxel have been shown to induce COX-2 and increase PGE_2 production by stimulating COX-2 transcription and stabilizing COX-2 messenger RNA (Subbaramaiah et al., 2003), possibly resulting in a disadvantage through affecting tumor growth, invasion and metastasis during anticancer therapy with taxanes. A combination therapy using taxanes and COX-2 inhibitors has recently been proposed (Olsen, 2005). However, the effect of taxanes on COX-2 expression in vascular smooth muscle cell remains unknown. Therefore, the aim of this concise study was to determine whether docetaxel affects interleukin (IL)-1β-induced COX-2 expression in smooth muscle cells isolated from rat aortas.

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2. Materials and methods

2.1. Cell preparation

Aortic smooth muscle cells (RASMCs) were prepared from 12-week-old male Wistar rats as described previously (Richman and Regan, 1998). The study protocols regarding treatment of animals were in accordance with the "Guidelines for Experiments Using Laboratory Animals in Hyogo College of Medicine". RASMCs were cultured in DMEM containing 10% FBS under 5% CO_2 at 37 °C. Cells were stimulated with IL-1β (10 ng/ml, R&D Systems, Minneapolis, MN, USA) for 24 h and then were harvested for immunoblot and immunocytochemical analyses.

2.2. Immunoblot analysis

RASMCs were collected with RIPA buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, a complete protease inhibitor mixture (Roche Applied Science), 1 mM Na_3VO_4 and 1 mM PMSF, followed by centrifugation at 8000 rpm for 10 min at 4 °C. Supernatant of the total cell extracts (20 μg) was subjected to SDS/PAGE. Immunoreactive bands for anti-COX-2 (Cayman Chemical; 1:1000), anti-ERK (Cell Signaling, Trask Lane, Danvers, MA, USA; 1:1000), anti-phospho-ERK (R&D Systems; 1:2000) and anti-α-smooth muscle actin (Sigma, 1 μg/ml) antibodies were visualized by ECL+Plus (GE Healthcare, UK).

2.3. Immunocytochemical analysis

After fixation by 2% paraformaldehyde containing 0.3% tritonX for 20 min, RASMCs were prepared for immunocytochemistry. Non-specific binding sites were blocked with 5% normal goat serum for 30 min. Cells were reacted with anti-COX-2 antibody (Cayman Chemical; 1:600). Immunoreactive sites for COX-2 were visualized by anti-rabbit IgG-Alexa488 (Molecular Probes, Eugene, OR, USA) and double-stained by anti-α-tubulin (Sigma, Saint Louis, MO, USA; 1:600), which was visualized by anti-mouse IgG-Alexa546 (Molecular Probes). Nuclear staining was performed with DAPI (Sigma). Confocal laser scanning microscopic observation was carried out using an LSM510 Meta (Carl Zeiss, Germany).

2.4. Chemicals

IL-1β (R&D Systems, Minneapolis, MN, USA) was dissolved in PBS, and 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene (U0126) (Promega, Madison, WI, USA) was dissolved in dimethylsulfoxide. These solutions were stored at −30 °C until use.

2.5. Statistical analysis

The data are shown as means ± S.E.M. Statistical analysis was done with Student's *t* test or analysis of variance followed by Scheffé's F-test as a post-hoc test. P values less than 0.05 were regarded as significant.

3. Results

3.1. Inhibition by docetaxel of COX-2 induction in RASMCs

Stimulation of RASMCs with IL-1β induced COX-2 expression, while COX-2 expression was not detected under a basal condition without any stimulants (Fig. 1A). Concomitant incubation of the cells with docetaxel (5–20 μg/ml) inhibited IL-1β-induced COX-2 expression in a concentration-dependent manner, while α-smooth muscle actin expression was not affected by the above concentrations of docetaxel (Fig. 1A and B). Docetaxel alone did not induce COX-2 expression under a basal condition (data not shown).

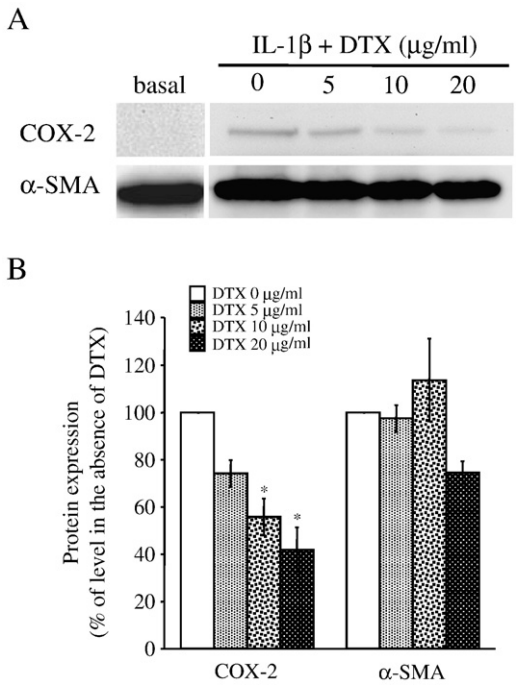

Fig. 1. Effects of docetaxel on protein expression of COX-2 and α-smooth muscle actin (α-SMA). COX-2 was induced by stimulation with IL-1β for 24 h in RASMCs in the presence or absence of docetaxel (0, 5, 10 and 20 μg/ml). A. Representative images of COX-2 and α-SMA immunoblots of RASMCs. B. Densitometric analysis of protein expression of COX-2 and α-SMA. DTX, docetaxel. Asterisks denote significant difference from the expression level in the absence of docetaxel (*, $P<0.05$).

3.2. No effect of docetaxel on ERK activity in RASMCs

Fig. 2A and B show effects of docetaxel on protein expression of ERK. Stimulation of RASMCs with IL-1β increased ERK activity as evaluated by phosphorylation of ERK, and this increased level of ERK activity was comparable in the absence and presence of docetaxel (20 μg/ml). Protein level of non-phosphorylated ERK, the reference of ERK activity, was not affected by stimulation with IL-Iβ and was comparable in the absence and presence of docetaxel (20 μg/ml). We confirmed that U0126, an ERK inhibitor, abolished both IL-1β-induced ERK activation and COX-2 expression (Fig. 2C).

3.3. Recovery of COX-2 expression in RASMCs after removal of docetaxel

Fig. 3A and B show effects of docetaxel and its removal on protein level of COX-2 induced by IL-1β in RASMCs. RASMCs showed a significant decrease in IL-1β-induced COX-2 expression after incubation with docetaxel (20 μg/ml) for 60 min. Then docetaxel was removed, and the cells were washed with a fresh medium and were further incubated with IL-1β for 24 h. After washing out of docetaxel, COX-2 expression in the cells was significantly augmented compared with that in the control cells stimulated with IL-1β for 60 min in the absence of docetaxel (Fig. 3A, upper; Fig. 3B, left). Protein level of α-smooth muscle actin in RASMCs was not affected by incubation with docetaxel and by washing out of it (Fig. 3A, lower; Fig. 3B, right).

Fig. 3C shows results of immunocytochemical analysis performed in order to reveal effects of docetaxel on expression of COX-2 and α-tubulin, which is known to be a target of the action of docetaxel for its anticancer effect. In RASMCs incubated with IL-1β in the absence of docetaxel (control), COX-2 immunoreactivity was detected in the nuclear membrane (arrowheads) and perinuclear cytoplasm (Fig. 3C, upper-left). Immunoreactivity for α-tubulin was detected as a ramified reticular pattern through the cytoplasm (Fig. 3C, lower-

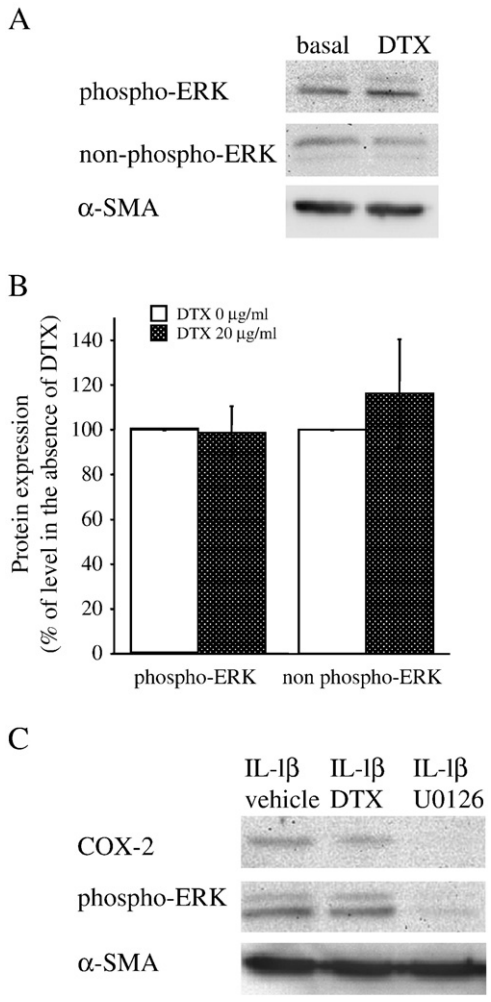

Fig. 2. A, B. Effects of docetaxel (DTX) on protein expression of phosphorylated and non-phosphorylated ERK. COX-2 was induced by stimulation with IL-1β for 24 h in RASMCs in the presence or absence of docetaxel (20 μg/ml). Representative images of ERK immunoblots of RASMCs (A) and densitometric analysis of protein expression of ERK (B). C. Effects of docetaxel (20 μg/ml) and U0126 (50 μM) on protein expression of COX-2 and phosphorylated ERK. α-smooth muscle actin (α-SMA) was used as an internal control.

left). In cells that had been treated with docetaxel for 24 h, COX-2 immunoreactivity was greatly decreased in both the nuclear membrane and cytoplasm (Fig. 3C, upper-center) compared with that in control cells (Fig. 3C, upper-left). In the presence of docetaxel, immunoreactivity for α-tubulin was detected as a thick bundle-like pattern (Fig. 3C, lower-center, arrows). This indicates polymerization of α-tubulin fibers, which is known as a mechanism for the anticancer action of docetaxel. In cells in which docetaxel had been washed out after incubation with IL-1β and docetaxel for 24 h and then had been incubated with IL-1β for a further 24 h, COX-2 immunoreactivity was regained in the same pattern (Fig. 3C, upper-right, arrowheads) as that in control cells without docetaxel incubation, while the corresponding expression of α-tubulin after washing out of docetaxel remained as a thick bundle-like pattern (Fig. 3C, lower-right, arrows). Merged images of immunostaining with COX-2 and α-tubulin indicate different intracellular distributions of COX-2 and α-tubulin (Fig. 3C, middle-left, center and right).

4. Discussion

This is the first study demonstrating that docetaxel inhibits COX-2 induction in vascular smooth muscle cells. In the present study, no effects of docetaxel on COX-2 level were found in RASMCs under a basal condition in the absence of IL-1β, although docetaxel has been shown to stimulate COX-2 induction in tumor cells (Subbaramaiah et al., 2003). The concentrations of docetaxel inhibiting COX-2 induction in RASMCs were comparable to the concentrations of docetaxel that have been reported to stimulate COX-2 induction in 184B5/HER cells (HER2/neu-transformed human mammary epithelial cells). Thus, docetaxel has diverse actions on COX-2, depending on the kind of cell. Taxanes including docetaxel exert antitumor actions through binding to polymerized tubulin to stabilize microtubules against disassembly. This action of docetaxel was also observed in RASMCs by immunocytochemical analysis. The stabilizing effect of docetaxel on microtubules of RASMCs was still observed at 24 h after docetaxel had been removed from the incubation medium of RASMCs following incubation with docetaxel for 24 h. On the other hand, expression of COX-2 in RASMCs stimulated with IL-1β had already recovered at 24 h after washing out of docetaxel. Thus, the inhibitory effect of docetaxel on COX-2 induction was reversible, and the duration of this docetaxel effect was shorter than duration of its effect on microtubules, namely, its anti-tumor effect. The IC_{50} values of docetaxel have been reported to be 0.13–2.5 ng/ml for various cancer cells (Hill et al., 1994). The concentrations (5–20 μg/ml) of docetaxel showing inhibitory action on COX-2 induction in RASMCs are much higher than the above IC_{50} values for cancer cells. These results indicate the possibility that the inhibitory action of docetaxel on COX-2 induction in vascular smooth muscle cells is only a toxic effect and is not clinically significant, although it is only possible to speculate on the concentration of docetaxel at loci of vascular smooth muscle cells being involved in tumor invasion and metastasis. Prostacyclin mediated by COX-2 in blood vessels is expected to work against tumor invasion and metastasis. Moreover, thromboembolic disease is a major complication in patients with cancer (Letai and Kuter, 1999), and prostacyclin plays a protective role in thrombosis. However, because of the short duration of the effect and relatively high concentrations showing the effect, it is unlikely that the inhibitory action of docetaxel on COX-2 expression influences local prostacyclin levels in blood vessels of patients treated with docetaxel.

ERK activation is known as a key upper-stream signal for IL-1β-stimulated COX-2 induction in RASMCs (Nakano and Wakabayashi, 2010). ERK has been shown to be activated by docetaxel in 184B5/HER cells (Subbaramaiah et al., 2003). However, docetaxel did not affect IL-1β-stimulated ERK activation in RASMCs. Thus, ERK activation is not involved in the inhibitory effect of docetaxel on COX-2 induction. As mentioned above, COX-2 induction in RASMCs was recovered at 24 h after washing out of docetaxel, while at this time point, tubulin depolymerization was still not observed in RASMCs. Therefore, the mechanism for docetaxel-induced inhibition of COX-2 expression is thought to be independent of the known anticancer action of docetaxel on microtubule stability. Moreover, the protein level of COX-2 was significantly higher in RASMCs at 24 h after washing out of docetaxel than in RASMCs stimulated with IL-1β in the absence of docetaxel. Although the reason for this rebound phenomenon of COX-2 expression by withdrawal of docetaxel is unknown, one possible explanation is that docetaxel acts on a pathway(s) (e.g., proteolysis pathway) besides the pathway for COX-2 synthesis. There is a possibility of docetaxel-induced decrease in proteolysis activity since tubulin is involved in proteolysis through intracellular protein transport. Further studies are needed to clarify the intracellular mechanisms for the actions of docetaxel on COX-2 induction in vascular smooth muscle cell.

In conclusion, docetaxel has an inhibitory action on IL-1β-stimulated COX-2 induction; however, the concentration of docetaxel required for this action is much higher than the concentration required for anticancer action.

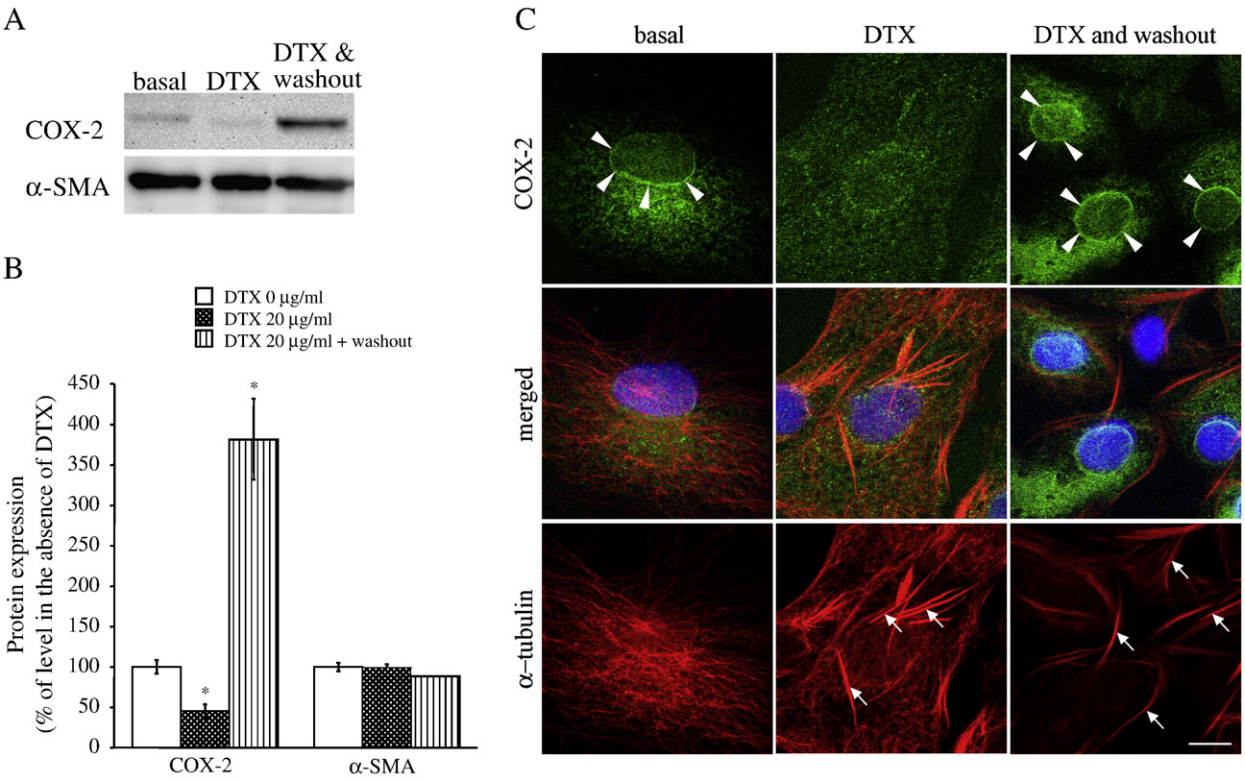

Fig. 3. Effects of treatment with docetaxel (DTX) and washout of docetaxel on protein expression of COX-2 and α-smooth muscle actin (α-SMA). COX-2 was induced by stimulation with IL-1β for 24 h in RASMCs in the presence or absence of docetaxel (20 μg/ml). In some cells, at 24 h after incubation with docetaxel, docetaxel was removed by washing out with the medium, and then the cells were further incubated with IL-1β and a vehicle of docetaxel for 24 h (washout). A. Representative images of COX-2 and α-SMA immunoblots of RASMCs. B. Densitometric analysis of protein expression of COX-2 and α-SMA. DTX, docetaxel. Asterisks denote significant difference from the expression level in the absence of docetaxel (*, $P<0.05$). C. Immunocytochemical analysis of COX-2 and α-tubulin expression in RASMC. RASMCs were stimulated with IL-1β (10 ng/ml) for 24 h in the absence (left column) or presence (central column) of docetaxel (10 μg/ml). Some cells were incubated in the presence of IL-1β for further 24 h after removal of docetaxel (right column). COX-2 immunoreactivity was greatly reduced by docetaxel treatment but recovered at 24 h after removal, whereas immunoreactivity for α-tubulin did not recover from polymerization due to docetaxel treatment. Immunoreactivity of COX-2 (arrowheads) and polymerized α-tubulin (arrows). Bar = 10 μm.

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