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The ecdysone inducible gene expression system: unexpected effects of muristerone A and ponasterone A on cytokine signaling in mammalian cells

Abstract

Inducible gene expression systems in mammalian cells have been shown to be valuable processes to study the specific function of a protein in differentiation, proliferation or survival/apoptosis. Usually, these systems use as inducible reagents, compounds that are thought to be neutral and devoid of physiological

or biologically undesirable effects in mammalian cells. We recently used the ecdysone inducible gene expression system in hematopoietic cells and found that the two inducer analogs of ecdysone, muristerone A and ponasterone A, altered the signaling pathways induced by IL-3 in the pro-B cell-line, Ba/F3. Indeed, we showed that these two analogs potentiate the IL-3-dependent activation of the PI 3-kinase/Akt pathway, which could ultimately interfere with the growth, and/or survival of these cells.

Full text

INTRODUCTION

Muristerone A and ponasterone A are two steroid insect hormones, analogs of ecdysone which is in- volved in the regulation of metamorphosis of *Drosophila melanogaster* [1]. Insect hormone responsiveness can be created in cultured mammalian cells by cotransfection of a functional ecdysone receptor and an ecdysone responsive promoter, driving the expression of the gene of interest [2]. Expression of this gene can be induced by muristerone A or ponasterone A in a dose-dependent manner, efficiently controlling the required amount of protein. In the ecdysone-inducible gene expression system (Invitrogen), the ecdysone receptor (EcR) from Drosophila and the RXR receptor are constitutively expressed from the vector pVgRXR, making a heterodimeric receptor, formed between the RXR and the EcR. In the presence of the inducers (muristerone A or ponasterone A), the ligand-receptor complex regulates expression of foreign genes placed under the control of a minimal promoter containing ecdysone response elements. The second advantage of the system is that ecdysteroids are thought to be neither toxic nor known to affect mammalian physiology, and appear therefore to be ideal candidates for use in cultured mammalian cells.

We are currently examining the signaling pathways induced by cytokines that control growth or survival of hematopoietic cells. We have identified several pathways that are crucial for these processes, including the PI 3-kinase/Akt and the JAK/STAT pathways [3]. In order to identify downstream targets of these pathways we wished to inducibly express dominant negative forms of several components of these signaling pathways by using the ecdysone inducible gene expression system. In a first set of experiments, we analyzed the effect of muristerone A and ponasterone A on the activity of the PI 3-kinase/Akt pathway

in Ba/F3 cells expressing the heterodimeric receptor (Ba/F3pVgRxR). We found surprisingly that both ecdysone analogs induced in presence of IL-3, the phosphorylation of Akt and its activation as judged by the phosphorylation of the pro-apoptotic Bad, a downstream target of Akt. Furthermore we showed by using the specific PI 3-kinase inhibitor, LY294002, that this induction required activation of PI 3-kinase, indicating that muristerone A and ponasterone A act synergistically with IL-3 to activate the PI 3-kinase/Akt pathway in these cells. Interestingly, the same results were obtained in the parental Ba/F3 cells, independently of the presence of a functional ecdysone receptor.

METHODS

Cell culture and reagents

Ba/F3pVgRxR cells were grown in RPMI 1640 medium (Life Technologies), containing 4% Wehi-3B supernatant as a source of IL-3, 10% fetal calf serum (Biological Industries), L-glutamine 2 mM (Life Technologies) and penicillin-streptomycin (10 U/ml and 10 mug/ml respectively; Life Technologies) at 37° C with 5% CO_2 (CM). LY294002 muristerone A and ponasterone A were purchased from Sigma and Invitrogen respectively.

Plasmids and transfections

The pVgRxR plasmid (Invitrogen), was electroporated in Ba/F3 cells (250 V, 960 muF). Cells were expanded for 24 hours before Zeocin selection. Pools of Zeocin-resistant cells were cloned by limiting dilution.

Western blotting

NP40 lysates were obtained by resuspending the cells in the following buffer: 1% NP40, 50 mM Tris pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 100 muM Na_3VO_4, 0.5 mM PMSF, 5 mug/ml aprotinin, 5 mug/ml leupeptin, 2 mug/ml pepstatin. Proteins were separated by electrophoresis on SDS PAGE and blotted onto a cellulose membrane (Hybond-C super membrane, Amersham Life Science). Blots were incubated as indicated with antibodies raised against P-Akt (Biolabs), Akt, Bad (Santa Cruz).

RESULTS AND DISCUSSION

The PI 3-kinase/Akt pathway has been shown to play an important role in the survival as well as in the regulation of cell cycle progression. Activation of PI 3-kinase by growth factors or cytokines, induces the phosphorylation and activation of the serine/threonine kinase Akt, which can in turn phosphorylate the Bcl-2 member Bad, thereby inhibiting its pro-apoptotic function and promoting cell survival [4, 5]. In order to use the ecdysone inducible gene expression system in the pro B cell line Ba/F3, we first analyzed the effects of muristerone A and ponasterone A on the activity of the PI 3-kinase/Akt pathway induced by IL-3 in Ba/F3pVgRxR cells. We first determined the expression and phosphorylation of Akt and Bad by immunoblot in extracts from Ba/F3pVgRxR cells growing in the presence of IL-3-containing medium (CM) (Figure 1A), in the absence (lane 1), or presence of muristerone A (5 muM) (lane 2). The results clearly show that muristerone A dramatically increased the phosphorylation of Akt on serine residue 473 (upper panel, lane 2) without any effect on Akt expression (middle panel). In a similar experiment, the expression and phosphorylation of Bad were measured by imunoblot (lower panel). IL-3 induces the appearance of two phosphorylated forms of Bad, BadP1 and BadP2, BadP2 expression being dependent on Akt activity [3]. As shown in Figure 1A, BadP1 and BadP2 were observed in the presence of IL-3 (lane 1), but the level of BadP2 protein expression was strongly increased after treatment with muristerone A (lane 2). These results indicate that muristerone A induced phosphorylation, as well as activation of Akt in Ba/F3pVgRxR. Similar results were also obtained with ponasterone A under the same conditions (data not shown).

To further analyze if muristerone A activates Akt/Bad proteins in a PI 3-kinase-dependent fashion, Ba/F3pVgRxR cells were cultured in the presence or absence of the specific PI 3-kinase inhibitor, LY294002, and then stimulated with muristerone A (5 muM) for 30 min (Figure 1B). We examined by immunoblot the phosphorylation status of Akt and Bad, and we observed that the addition of LY294002 inhibited the effect of muristerone A on Akt (lane 2, upper panel) and Bad phosphorylation (lane 2, lower panel). Collectively, these results indicate that muristerone A or ponasterone A efficiently activate the PI 3-kinase/Akt pathway in Ba/F3pVgRxR cells, in the presence of IL-3. When similar experiments were conducted in the absence of IL-3, we failed to detect a muristerone A-dependent phosphorylation of Akt and Bad. Interestingly, IL-3 (CM) was also required for the induction of expression of a foreign gene in Ba/F3 cell lines established with the ecdysone inducible gene expression system (data not shown). Thus, our data indicate that muristerone A or ponasterone A stimulate

the IL-3-dependent activation of the PI 3-kinase/Akt pathway. Unexpectedly, muristerone A and ponasterone A activated the Akt/Bad proteins in a PI 3-kinase-dependent fashion, in the parental Ba/F3 cells, independently of the presence of a functional ecdysone receptor (data not shown).

The experiments described above were performed with 5 muM of muristerone A or ponasterone A but, we also observed a dose-dependent activation of the PI 3-kinase/Akt pathway with these inducing reagents ranging from 1 to 10 muM (data not shown). This range of concentration was even below the range needed to induce the expression of a foreign gene in established Ba/F3 cell lines (data not shown).

CONCLUSION

In conclusion, our data clearly indicate, that these ecdysone analogs have important effects on cytokine signaling in hematopoietic cell lines that should be carefully controlled when the role of signaling proteins in cell growth and survival needs to be analyzed. For this reason, the use of the ecdysone-inducible gene expression system must take into consideration these secondary effects and their potential consequences in the interpretation of any results.

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Figures

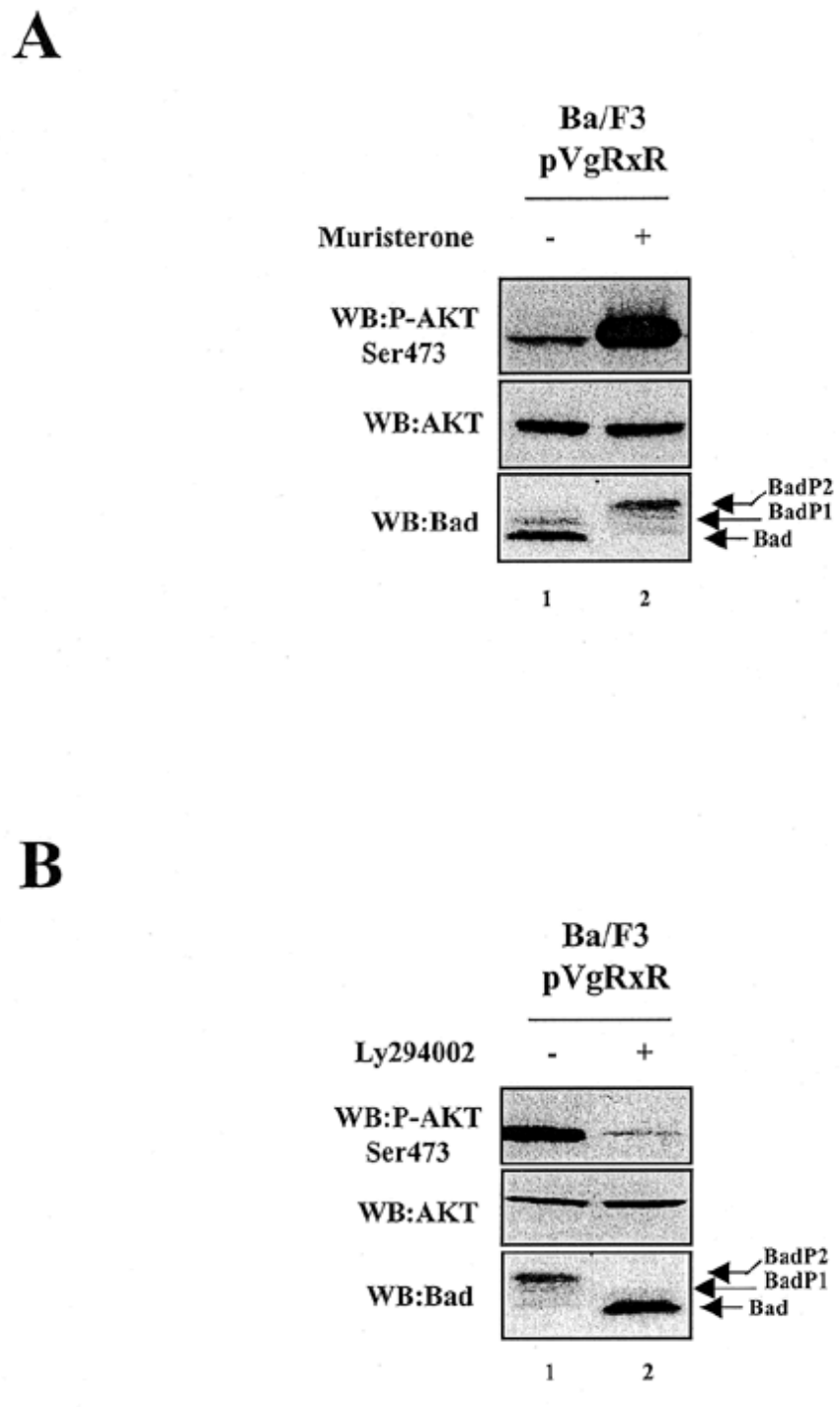


Figure 1

A: Muristerone A induces phosphorylation of Akt and Bad in Ba/F3 cells.

Extracts from Ba/F3pVgRxR cells growing in CM, in the absence (lane 1) or presence of muristerone A 5 muM for 30 min (lane 2), were prepared and analyzed by Western blot, with an anti-P-AKT antibody (upper panel) or with an anti-Akt antibody (middle panel). A similar experiment was performed to analyze Bad phosphorylation with an anti-Bad antibody (lower panel). Positions of unphosphorylated Bad, BadP1 and BadP2 are indicated. **B:** Effect of Ly294002 on the phosphorylation of Akt and Bad after muristerone A induction. Ba/F3pVgRxR cells were cultured in CM, in the absence (lane 1) or presence of Ly294002 50 muM for 8 hours (lane 2), and then stimulated with muristerone A 5 muM for 30 min (lanes 1, 2). Cell extracts were prepared and analyzed by Western blot, with an anti-P-Akt antibody (upper panel) or with an anti-Akt antibody (middle panel). A similar experiment was performed to analyze Bad phosphorylation with an anti-Bad antibody (lower panel). Positions of unphosphorylated Bad, BadP1 and BadP2 are indicated.

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