Essential role of p21/waf1 in the mediation of the anti-proliferative effects of GHRH antagonist JMR-132

Aspasia-Athina Volakaki, Daniel Lafkas, Eva Kassi, Andrew V Schally1,2, Athanasios G Papavassiliou and Hippokratis Kiaris

Department of Biological Chemistry, University of Athens Medical School, 75 M. Asias Street, 11527 Athens, Greece

1Research Service (151), VA Medical Center, Miami, Florida 33125, USA
2Division of Hematology/Oncology, Departments of Pathology and Medicine, University of Miami Miller School of Medicine, Miami, Florida 33101, USA

(Correspondence should be addressed to H Kiaris; Email: hkiaris@med.uoa.gr)

Abstract

GHRH, besides its neuroendocrine action in controlling the release of GH from the pituitary, stimulates the growth of various cancers in vivo and in vitro by direct mechanism(s). However, the molecular mechanism that mediates these proliferative effects of GHRH in extrapituitary tissues remains poorly characterized. In the present study, we investigated whether the tumor suppressor p21/waf1 is involved in the mediation of the proliferative effects of GHRH in A549 human lung cancer epithelial cells. Exposure of A549 cells to the GHRH antagonist JMR-132 caused a significant inhibition in the rate of cell proliferation. In A549 cells, GHRH suppressed while JMR-132 increased the levels of p21 expression in a dose-dependent manner. This suggests that GHRH could regulate p21 levels. We then evaluated whether p21 is required in A549 cells for the regulation of cell proliferation by GHRH. To this end, we knocked-down p21 expression in A549 cells by siRNA and assessed the effects of antagonist JMR-132 on cell proliferation. We found that the loss of p21 expression abolished the anti-proliferative effects of JMR-132. Suppression of p21 expression by siRNA in human HT29 colon cancer cells and non-transformed mouse osteoblasts KS483 also blocked the anti-proliferative effects of JMR-132 suggesting that the regulation of cell proliferation by GHRH is p21 dependent. These results shed light on the molecular mechanism of action of GHRH antagonists in tumor tissues and suggest that the antineoplastic activity of GHRH antagonists could be considered for the treatment of cancers expressing p21.

Journal of Molecular Endocrinology (2008) 41, 389–392

Introduction

The hypothalamic neuropeptide growth hormone-releasing hormone (GHRH) stimulates the production and release of GH in the pituitary (Kiaris et al. 2005). Besides the hypothalamus, GHRH is produced by various extrapituitary tissues as well as by cancers in which it operates as a stimulator of cell proliferation. Consequently, inhibition of action of GHRH by specific antagonistic peptide analogs may emerge as a novel anticancer strategy (Schally et al. 2001, Kiaris et al. 2003a, b, 2005, Schally et al. 2008). However, despite a well-documented expression of GHRH in various primary and experimental human cancers and cell lines, as well as the clearly demonstrated antineoplastic activity of GHRH antagonists in vitro and in vivo, the knowledge about the mechanism of GHRH action at the subcellular level is limited. The major receptor that mediates the effects of GHRH in the extrapituitary tissues is SV1, a splice variant of the pituitary GHRH receptor that also has ligand-independent activity (Rekasi et al. 2000, Kiaris et al. 2002, Barabutis et al. 2007). With regard to the effects of GHRH on proliferation, at the level of the signaling cascades activated upon binding of GHRH to its receptor(s), a role for mitogen-activated protein kinase (MAPK), and recently Pit-1 transcription factor has been recognized in pituitary cells (Pombo et al. 2000, Solloso et al. 2008). In breast cancer cells, Ras, Raf, and MAPK have been shown to play an essential role in the mediation of the proliferative effects of GHRH (Sirwardana et al. 2006). It has also been shown that in MCF-7 breast cancer cells, upon binding of GHRH to SV1 receptor, the increase in cell proliferation is accompanied by elevated expression of cyclin D1, an important cell-cycle regulator (Barabutis et al. 2007).

In the present study, we investigated the role of p21 in mediating the effects of GHRH in the proliferation of A549 human lung cancer epithelial cells, HT29 human colon cancer cells, and mouse non-transformed osteoblasts KS483. p21 is a cycle-dependent kinase inhibitor that plays a major role in the regulation of cell cycle and has been recognized as a direct target of p53 tumor suppressor gene (el-Deiry et al. 1993, Weiss 2003). While p21 is rarely mutated in primary human cancers, it is frequently characterized by aberrant expression.
Materials and methods

Peptides

Human GHRH (hGHRH) (1-29)-NH$_2$ was obtained from Dr Parlow (National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, USA). The synthesis of the GHRH antagonist JMR-132 [PhAc$^6$,D-Arg$^2$,Phe(4-l)$^6$,Ala$^8$,Har$^9$,Tyr(Me)$^{10}$,His$^{11}$,Abu$^{15}$,His$^{20}$,Nle$^{27}$,D-Arg$^{28}$,Har$^{29}$] hGHRH(1-29)-NH$_2$, where Abu is a-aminobutyric acid, Har is homoarginine, Nle is norleucine, PhAc is phenylacetyl, and Tyr(Me) is O-methyltyrosine was reported previously (Buchholz et al. 2007).

Cell culture

A549 human lung alveolar and HT29 human colon cancer cells were obtained from American Type Culture Collection (Manssas, VA, USA) and maintained in DMEM containing 10% fetal bovine serum (FBS) and antibiotics/antimycotics. KS483 cells (provided by Dr Karperien, The Netherlands) were grown in a-MEM Phenol Red free medium supplemented with 10% FBS DCC treated. Tissue culture reagents were obtained from Invitrogen. The rate of cell proliferation was assessed after exposure of cells for 48 h to GHRH or JMR-132 at concentrations indicated and the cells being counted under inverted microscope after standard trypan blue exclusion assay. Experiments have been performed in triplicates and average values are shown.

siRNA and western blot analysis

Suppression of p21 in A549 and HT29 cells, or KS483 cells, was achieved by transfecting cells with siRNA specific for human or mouse p21 (Ambion, Austin, TX, USA) respectively according to the manufacturer’s instruction using Lipofectamine 2000 reagent. Forty eight hours post-transfection cells were lysed by using RIPA reagent and total protein was subjected to western blot analysis. Antibodies for p21 and actin were obtained from Sigma. All experiments have been performed at least three times independently and similar results were obtained.

Statistical analysis

Statistical analysis was performed with the Student’s t-test and results were considered significant when P<0.05.

Results and discussion

Initially, we evaluated the effects of GHRH and JMR-132 on the proliferation of A549 cells. As depicted in Fig. 1, the exposure of A549 cells to JMR-132 at 10$^{-6}$ M for 48 h resulted in 35% (P<0-05) decrease, while GHRH caused about 10%, yet insignificant, stimulation on the rate of cell proliferation suggesting that these cells are sensitive to JMR-132 treatment. Consistent with previous findings on the effects of GHRH antagonists on cell proliferation, no evidence of cytotoxicity was noted in the cells exposed to JMR-132.

Subsequently, we evaluated the effects of these peptides on the expression of the tumor suppressor gene p21/waf1. A549 cells were exposed to GHRH or JMR-132 at 10$^{-10}$ M, 10$^{-8}$ M and 10$^{-6}$ M and the expression of p21 was assessed by western blot analysis. As shown in Fig. 2, increasing concentrations of GHRH caused a dose-dependent reduction while JMR-132 produced an elevation in the expression of p21. This is consistent with the effects of these peptides on the proliferation of A549 cells. It is noted, however, that whereas GHRH caused a considerable reduction in p21 levels, its effects on cell proliferation were relatively small when compared with those of JMR-132, implying that p21 levels might not be the limiting factor for the increase in cell proliferation elicited by GHRH. Furthermore, JMR-132 stimulated p21 expression at 10$^{-10}$ to 10$^{-6}$ M while it inhibited cell proliferation only at 10$^{-6}$ M. Probably this is due to the fact that p21 induction is necessary but not sufficient for cell proliferation inhibition, or that a certain threshold in p21 expression levels should be achieved in order to cause inhibition of cell proliferation.

To determine whether these findings are causatively associated with the anti-proliferative effects of JMR-132 or reflect the reduced proliferation of A549 cells, we knocked down p21 expression by siRNA and then evaluated if JMR-132 was capable of suppressing A549 proliferation. As shown in Fig. 3, in the absence of p21, JMR-132 at 10$^{-6}$ M lost its ability to inhibit A549 cell proliferation, indicating that this cell-cycle regulator is required for the suppression of cell proliferation by antagonistic analogs of GHRH. For this experiment, we used siRNA for p21 at 5 nM, because at higher concentrations siRNA for p21 exerted toxic effects (Fig. 3a and unpublished observations).
In order to test whether our findings represent a more general mechanism of GHRH action or they are limited to A549 lung cancer cells we evaluated the consequences of p21 knock-down in cells of diverse origin, such as human colon cancer cells and mouse non-transformed osteoblasts KS483. In HT29 cells, we found that JMR-132 stimulated p21 expression at $10^{-6}$ M–$10^{-10}$ M, notwithstanding the fact that stimulation was slightly more potent at $10^{-8}$ M (Fig. 4). Subsequently, we knocked-down p21 by siRNA and tested the effects on cell proliferation, of JMR-132 at $10^{-8}$ M alone or in combination with p21 siRNA at 10 nM. As shown in Fig. 4, suppression of p21 expression significantly inhibited the anti-proliferative effects of JMR-132 ($P<0.01$). Similar results were obtained with KS483 with the exception that in these cells, while treatment with JMR-132 $10^{-10}$ M had no considerable effect on p21 expression (not shown), it effectively blocked the suppression of p21 caused by estradiol at $10^{-7}$ M. Furthermore, siRNA for p21 at 5 nM significantly inhibited the anti-proliferative effects of JMR-132 in the presence of $10^{-7}$ M estradiol (Fig. 5). Therefore, we conclude that modulation of p21 expression by GHRH antagonists likely represents a general mechanism for the action of these agents in cell growth.
In none of the cells used above, siRNA for p21 alone did not affect cell proliferation considerably, and scrambled siRNA also did not affect p21 expression (data not shown).

Thus, considering previous findings on the mechanism of action of GHRH analogs in extrapituitary tissues, the interaction of GHRH with its membrane receptors, may initiate a signaling cascade that involves Ras, Raf, and MAPK and which eventually results in the modulation of p21 expression in the nucleus. This mechanism of modulation of p21 expression appears to play a major role in the mediation of the effects of GHRH analogs on cell proliferation.

These results shed light on the mechanism of action of GHRH antagonists by linking directly GHRH to the cell-cycle machinery, indicating that p21 is a critical target of GHRH analogs. Our results also imply that the GHRH antagonists should be considered particularly for the therapy of cancers that express p21 activity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the grant 05NONEU-13 from GSRT, and Kapodistrias 06, ELKE, University of Athens, Greece.

Acknowledgements

We thank Dr Karperien (Leiden University, The Netherlands) for the KS483 cells.

References


