Special Issue

Evidence that phosphatidylcholine-specific phospholipase C is a key molecule mediating insulin-induced enhancement of gene expression from human cytomegalovirus promoter in CHO cells

Yingpei Zhang, Yoshinori Katakura, Perry Seto & Sanetaka Shirahata

Graduate School of Genetic Resources Technology, Kyushu University, Hakozaki 6–10–1, Higashi-ku, Fukuoka 812–81, Japan

Received and accepted June 1996

Key words: Chinese hamster ovary cell, human cytomegalovirus promoter, insulin, phosphatidylcholine-specific phospholipase C, signal transduction

Abstract

The signal transduction from insulin to its receptors and Ras has been extensively studied, while little has been reported beyond these steps. We found that the expression of human interleukin 6 gene under the control of immediate early gene promoter of human cytomegalovirus was enhanced by insulin stimulation in Chinese hamster ovary cells. The induction effect of insulin was not significantly affected by inhibitors or activators of conventional protein kinase C, cAMP dependent protein kinase and phosphoinositide -3 kinase, however, pre-incubation of the cells with D609, a specific inhibitors of phosphatidylcholine-specific phospholipase C completely abolished the induction effect. These results clearly demonstrate that phosphatidylcholine-specific phospholipase C is a key molecule mediating insulin-induced enhancement of hIL-6 expression from the human cytomegalovirus promoter in Chinese hamster ovary cells and strongly suggest that it plays an important role in the insulin signaling pathways.

Abbreviations: CHO – Chinese hamster ovary; hCMV promoter – immediate early gene promoter of human cytomegalovirus; hIL-6 – human interleukin 6; PC-PLC-phosphatidylcholine-specific phospholipase C; PI-3 kinase – phosphoinositide 3 kinase; PKA – cAMP dependent protein kinase; PKC – protein kinase C.

Introduction

Insulin is a major physiological regulator of many metabolic pathways, including protein synthesis, lipogenesis and glucose utilization. These biological actions of insulin are initiated by activation of the heterotetrameric receptor which possesses tyrosine kinase activity (Kasuga *et al.*, 1983), leading to rapid autophosphorylation of receptor tyrosine residues as well as the tyrosine phosphorylation of other cellular substrates (Sun *et al.*, 1991). Other early cellular events initiated by insulin stimulation includes increase of phosphatidylinositol (PI)-3 kinase activity and the amount of the active Ras. GTP and activation of numerous protein serine/threonine kinase (Sakaue

et al., 1995, Yonezawa *et al.*, 1994). These early signaling events have been extensively studied recently, however little has been reported on the signaling further downstream of these steps. We have previously found that the immediate early gene promoter of human cytomegalovirus (hCMV) was activated by Ras oncoproteins in Chinese hamster ovary (CHO) cells (Seto *et al.*, 1996). Since insulin stimulation will result in activation of Ras, we expected that the expression of the reporter gene under the control of hCMV promoter will be enhanced by insulin stimulation as a result of activation of Ras and other possible signaling pathways. Because the hCMV promoter contains putative binding sites of many transcriptional factors including NF- κ B, CREB, AP-1, AP-2, SP-1, SRF, the activity

of hCMV promoter should be affected by many cellular signals. Here we report that the insulin stimulation enhanced the expression of the reporter gene regulated by hCMV promoter, and the induction effect of insulin was completely abolished by the pretreatment of the cells with D609 (tricyclodecan-9-yl xanthogemate K), a specific inhibitor of phosphatidylcholine-specific phospholipase C (PC-PLC). We also provide evidence showing that conventional protein kinase C (PKC), cAMP dependent protein kinase (PKA) and PI-3 kinase are not key molecules mediating the induction of insulin, although their activity is required for efficient expression of the reporter gene.

Materials and methods

Cell line

A recombinant CHO cell line A7, producing human interleukin 6 (hIL-6) was established as described previously (Seto *et al.*, 1996). Briefly, *dhfr* deficient CHO DUKBX11 cells were co-transfected with a *hIL-6* expression vector pCMVP-*IL6* and pSV2/*bsr*. Stable transformants were selected in 5 μ g/ml of Blasticidin S and clone A7 which demonstrated the highest productivity was isolated. A7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with hypoxanthine (13.9 μ g/ml), thymidine (3.9 μ g/ml) and 5% fetal bovine serum (FBS) in 5% CO₂ at 37 °C.

Reagents

D609, wortmannin, dibutyl-*c*-AMP, H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine 2HCl) and calphostin C were purchased from BIOMOL (Plymouth Meeting, PA). H89 (N-(2-(p-bromocinnamylamino)ethyl)-5-isoquinolinesulfonamide 2HCl) and PMA (phorbol 12-myristate 13-acetate) were purchased from GIBCO BRL (Gaithersburg, MD). PC-PLC was purchased from Boehringer Mannheim (Indianapolis, IN). The insulin was purchased from Novo Nordisk (Bagsvaerd, Denmark).

Protocol

The A7 cells were plated in 24 well plates at 3×10^4 cells/well and allowed to grow for 12 h in DMEM media supplemented with 5% FBS. The cells were washed twice with phosphate buffered saline (PBS),

then incubated for 12 h in DMEM media without serum. After again washing twice with PBS, the cells were treated with various reagents in serum-free media for 2 h as described in the legends of figures. The reagents were prepared just prior to use by diluting the stock solutions with medium. Distilled water or DMSO, which was used to dissolve these reagents, were used as control and were found to have no effect on the hIL-6 production. The effects of these reagents were evaluated by quantitating the amount of hIL-6 secreted into the supernatant with the sandwich enzyme-linked immunosorbent assay (sandwich ELISA) (Yano *et al.*, 1994).

Results

We first examined whether insulin stimulation of the hCMV promoter in recombinant CHO cells would enhance the expression of the reporter gene. When the serum-starved A7 cells were stimulated with insulin, a significant enhancement of hIL-6 expression was observed (data included in every figure), although the degree of induction by insulin slightly varied in separate experiments.

Since insulin stimulation also activates phosphoinositide (PI)-3 kinase (Sakaue *et al.*, 1995), we used wortmannin, an inhibitor specific for PI-3 kinase, to study whether it participates in the insulin-induced enhancement of hIL-6 expression. As shown in Figure 1A, both basal and insulin-induced hIL-6 expression were repressed, but the inducibility by insulin was not abolished. These results demonstrate that PI-3 kinase is not the key molecule involved in mediating the insulin-induced enhancement of hIL-6 expression, although the signaling pathway via PI-3 kinase is needed for efficient expression of hIL-6.

It has been reported that insulin signaling is regulated by intracellular levels of cAMP, probably through cAMP-dependent PKA (Burgering *et al.*, 1993). There are four binding sites of CREB in the hCMV promoter which can be activated by PKA. To investigate the role of PKA in insulin-induced enhancement of hIL-6 gene expression from hCMV promoter, we treated the A7 cells with the PKA potent inhibitor H89. As shown in Figure 1B, H89 greatly inhibited the basal and insulin-induced hIL-6 expression. The relative inducibility by insulin was, however, not significantly affected. We then treated the cells with dibutyl *c*-AMP, an activator of PKA. As shown in Figure 1C, dibutyl *c*-AMP slightly affected the basal expression,

Figure 1. Effects of specific inhibition or activation of PI-3 kinase, PKA and PKC on the insulin-induced enhancement of hIL-6 production. A7 cells (3×10^4 cells) were plated into 24 well plates and allowed to grow overnight in DMEM media supplemented with 5% FBS. The cells were washed twice with PBS, then incubated in serum-free DMEM media overnight. After washing twice with PBS, the cells were treated with various concentrations of specific inhibitors or activators for 15 min, then stimulated with insulin (1 $\mu\text{g/ml}$) ($\square$) or distilled water as control ($\blacksquare$). The cells were further incubated for two hours. The concentrations of hIL-6 in the supernatants were quantitatively determined using sandwich ELISA. All experiments were done in triplicate and average values were shown with error bars indicating standard errors. A, wortmannin; B, H89; C, dibutyryl cAMP; D, calphostin C; E, H7; F, PMA.

but the insulin-induced hIL-6 expression did not proportionally increased as expected. These results suggest that the role of PKA in insulin-stimulated cells is complicated, and clearly show that PKA is not the key molecule mediating the insulin-induced enhancement of hIL-6 expression in this cell line.

A7 cells were then treated with calphostin C, a PKC specific inhibitor, to study the role of PKC. As shown in Figure 1D, both basal and insulin-induced hIL-6 expression were significantly repressed, however, the inducibility by insulin was not abolished. Similar results were also obtained when using H7, an inhibitor specific both for PKC and PKA (Figure 1E). Pretreatment with the PKC activator PMA greatly increased both basal and insulin-induced hIL-6 expression (Figure 1F). These results demonstrate that the signaling pathway via PKC is very important for activation of the hCMV promoter irrespective of insulin stimulation.

When these CHO cells were treated with D609, a specific inhibitor of PC-PLC, the inducibility by insulin was completely blocked (Figure 2A). The hIL-

Figure 2. PC-PLC and insulin-induced enhancement of hIL-6 production. Experimental conditions were the same as in Figure 1, except for the treatment of cells with various concentrations of reagents for 15 min. The cells were treated with various concentrations of D609 for 15 min, then stimulated with insulin (1 $\mu\text{g/ml}$) ($\square$) or distilled water as control ($\blacksquare$) (A and B). Cells were treated with D609 for 15 min, then stimulated with insulin (1 $\mu\text{g/ml}$) ($\square$), 10% serum (hatched) or distilled water as control ($\blacksquare$) (C). Without the pretreatment of with D609, cells were stimulated with insulin (1 $\mu\text{g/ml}$) ($\square$), PC-PLC (hatched) or distilled water ($\blacksquare$) (D).

6 production was severely repressed in the presence of high concentrations of D609 (Figure 2B), however this repression was not a result of cytotoxic effect of D609, because serum-stimulation of the D609-pretreated cells could still induce a significant increase in hIL-6 production (Figure 2C). To confirm that PC-PLC participates in the insulin-induced enhancement of hIL-6 production, we treated cells with an exogenous PC-PLC derived from bacteria. As shown in Figure 2D, a moderate increase in hIL-6 production was observed. Taken together, these results clearly demonstrate that PC-PLC is a key molecule mediating insulin-induced enhancement of hIL-6 expression from hCMV promoter, and strongly suggest that PC-PLC plays an important role in insulin signaling network.

Discussion

Because the hCMV promoter of the pRc/CMV vector (Invitrogen, San Diego, CA) possesses the puta-

tive binding sites of many transcriptional factors such as NF- κ B (-420, -268, -163, -100, as referred to the transcription initiation site), CREB (-460, -407, -324, -138), AP-1 (-171), AP-2 (-471), (-104), SP-1(-54) and SRE (-536), it might be reasonable to expect that multiple transcriptional factors can be activated in our recombinant CHO cell lines by insulin stimulation, which may result in the enhanced expression of recombinant hIL-6. However, the insulin-induced enhancement of hIL-6 expression was completely abolished by inhibition of PC-PLC. This suggests that PC-PLC occupies a key position in the signal transduction pathways from insulin the hCMV promoter.

Although several reports showing the importance of PC-PLC in signal transduction pathway have recently been published (Johansen *et al.*, 1994; Arenzana-Seisdedos *et al.*, 1993), the signaling pathway via PC-PLC has not been well characterized. However, PC-PLC mediated PC hydrolysis seemed to be stimulated by Ras, and has been reported to couple with Ras to activate Raf-1 in certain cell types, therefore, PC-PLC has been positioned downstream of Ras and upstream of Raf-1 in mitogenic signal transduction (Cai *et al.*, 1992; Cai *et al.*, 1993). Since Ras proteins are activated by insulin stimulation, our results imply that the signal transduction from Ras to hCMV promoter were also blocked by D609. This suggests that PC-PLC might play an important role in Ras induced activation of hCMV promoter in CHO cells observed previously (Seto *et al.*, 1996).

Recently it has been reported that the increase PC-PLC mediated hydrolysis of PC induces functional activation of NF- κ B in human monocytes and T lymphocytes (Arenzana-Seisdedos *et al.*, 1993). Because there are four binding sites of NF- κ B in hCMV promoter, it is possible that D609 blocks the induction effect of insulin via inhibiting the PC-PLC-mediated activations NF- κ B. Whether NF- κ B and other transcriptional factors are inhibited by D609 remains to be elucidated.

References

- Arenzana-Seisdedos F, Fernandez B, Dominguez I, Jacque JM, Thomas D, Diaz-Meco MT, Moscat J and Virelizier JL (1993) Phosphatidylcholine hydrolysis activates NF- κ B and increases human immunodeficiencyvirus replication in human monocytes and T lymphocytes. *Mol Cell Biol* 67: 6596–6604.
- Burgering BMT, Pronk GJ, van Weeren PC, Chardin P and Bos JL (1993) CAMP antagonizes p21^{ras}-directed activation of extracellular signal-regulated kinase 2 and phosphorylation of mSos nucleotide exchange factor. *EMBO J* 12: 4211–4220.
- Cai H, Erhardt P, Szeberenyi S, Diaz-Meco MT, Johansen T, Moscat J and Cooper GM (1992) Hydrolysis of phosphatidylcholine is stimulated by Ras proteins during mitogenic signal transduction. *Mol Cell Biol* 12: 5329–5335.
- Cai H, Erhardt P, Troppmair J, Diaz-Meco MT, Sithanandam G, Rapp UR, Moscat J and Cooper GM (1993) Hydrolysis of phosphatidylcholine couples Ras to activation of Raf protein kinase during mitogenic signal transduction. *Mol Cell Biol* 13: 7645–7652.
- Johansen T, Bjorkoy G, Overvatn A, Diaz-Meco MT, Traavik T and Moscat J (1994) NIH3T3 cells stably transfected with the gene encoding phosphatidylcholine-hydrolyzing phospholipase C from *Bacillus cereus* acquire a transformed phenotype. *Mol Cell Biol* 14: 646–654.
- Kasuga M, Fujita-Yamaguchi Y, Blithe D and Kahn, CR (1983) Tyrosine specific-protein kinase activity is associated with the purified insulin receptor. *Proc Natl Acad Sci USA* 80: 2137–2141.
- Sakaue H, Hara K, Noguchi T, Matozaki T, Kotani K, Ogawa W, Yonezawa K, Waterfield MD and Kasuga M (1995) Ras-independent and wortmannin-sensitive activation of glycogen synthase by insulin in Chinese hamster ovary cells. *J Biol Chem* 270: 11304–11309.
- Seto P, Katakura Y, Zhang Y, Inoue Y, Teruya K, Miura T, Ohashi H and Shirahata S (1996) Enhancement of recombinant protein productivity in Chinese hamster ovary cells by oncogenes. In: Funatsu K *et al.* (ed.) *Animal Cell Technology: Basic and Applied Aspects*. Volume 8, Kluwer Academic Press, Dordrecht, The Netherlands (in press).
- Sun XJ, Rothenberg P, Kahn CR, Backer JM, Araki E, Wilden PA, Cahill DA, Goldstein BJ and White MF (1991) Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature (London)* 352: 73–77.
- Yano T, Teruya K, Shirahata S, Watanabe J, Osada K, Tachibana H, Ohashi H, Kim E-H and Murakami H (1994) Ras oncogene enhances the production of a recombinant protein regulated by the cytomegalovirus promoter in BHK-21 cells. *Cytotechnology* 16: 167–178.
- Yonezawa K, Ando A, Kaburagi Y, Yamamoto-Honda R., Kitamura T, Hara K, Nakafuku M, Okabayashi Y, Kadowaki T, Kaziro Y and Kasuga, M (1994). Signal transduction pathways from insulin receptors to Ras. *J Biol Chem* 269: 4634–4640.

Address for Correspondence: Yingpei Zhang, Laboratory of Cellular Regulation Technology, Graduate School of Genetic Resources Technology, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-81, Japan.