Hypoglycemia-induced c-Jun Phosphorylation Is Mediated by c-Jun N-terminal Kinase 1 and Lyn Kinase in Drug-resistant Human Breast Carcinoma MCF-7/ADR Cells*

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We studied the signal transduction mechanism that is involved in c-Jun phosphorylation evident after glucose deprivation in MCF-7/ADR cells. Glucose deprivation caused an immediate increase in tyrosine phosphorylation in MCF-7/ADR cells and specifically activated Lyn kinase, a src family tyrosine kinase. In addition, hypoglycemic treatment strongly activated c-Jun N-terminal kinase 1 (JNK1), leading to the phosphorylation and activation of c-Jun. Experiments with Lyn antisense oligonucleotides demonstrated that Lyn kinase activation was responsible for the activation of JNK1 but not extracellular signal-regulated kinase. We also observed glucose deprivation-induced Ras activation in MCF-7/ADR cells. These results indicate a possible Ras-dependent signaling pathway involving Lyn kinase and JNK1, which leads to the glucose deprivation-induced responses in MCF-7/ADR cells.

The initiation of angiogenic activity is recognized as one of the critical steps in tumor development and metastasis. It has been demonstrated that ischemia/hypoxia (1, 2), radiation (3, 4), or tumor promotors (5) can stimulate the synthesis of angiogenic factor(s), such as basic fibroblast growth factor (bFGF)1 and vascular endothelial growth factor. Angiogenic factors can trigger the formation of new blood vessels, allowing more nutrients and O2 to reach cancer cells, and also provide a route for metastasis. A fundamental question that remains unanswered is how the stresses stimulate the synthesis of these angiogenic factors.

The genes encoding angiogenic factors such as bFGF have been shown to contain AP-1 cis-acting elements (12-O-tetradecanoylphorbol-13-acetate response element) in their promoter region (6–8), which are recognized by AP-1 transcription factors (Jun and Fos family proteins) (9, 10). The activity of AP-1 transcription factors is controlled by transcriptional and post-transcriptional regulation. It has been shown that transcriptional activation of the c-jun gene is exerted through the distal and proximal AP-1 binding sites (9), which are recognized by either c-Jun homodimers or c-Jun/activating transcription factor-2 heterodimers (9, 10). The transcriptional activity of these binding factors is stimulated by the phosphorylation of c-Jun (11–13), and possibly activating transcription factor-2 (14, 15), by c-Jun N-terminal kinase (JNK). The induction of c-fos transcription is mediated by the serum response element (SRE) and the v-sis conditioned medium induction element. The SRE is recognized by a homodimer of serum response factor (16), and the binary SRE-serum response factor complex interacts with the ternary complex factor (TCF) (17). It has been demonstrated that the activity of TCF is rapidly increased in response to stimulation with various agents, such as growth factors, which leads to the activation of extracellular signal-regulated kinase (ERK). ERK has been shown to be responsible for the phosphorylation and activation of TCF (18, 19). Taken together, phosphorylation of c-Jun and other transcription factors appears to be the link between the transcriptional activity of AP-1 containing genes and ERK or JNK kinase signaling cascades, allowing the expression of these genes to be triggered by different stimuli.

We have previously demonstrated that hypoglycemia activates the expression of c-jun and c-fos genes, which results in an increase in AP-1 activity and subsequent bFGF gene expression in drug-resistant human breast carcinoma MCF-7/ADR cells (20). In an effort to fully understand the signaling mechanism that triggered these responses, we studied the phosphorylation events that may lead to the activation of AP-1 transcription factors. The study reported here demonstrates that hypoglycemic treatment of MCF-7/ADR cells activated Lyn kinase, which increased the activity of JNK1 and the phosphorylation of c-Jun. These results suggest a possible signal transduction pathway in MCF-7/ADR cells that responds to hypoglycemic conditions.

**MATERIALS AND METHODS**

*Construction of JNK1 Expression Vector—The expression vector pCMV5-JNK1 (21) was kindly provided by Dr. M. Karin (University of California, San Diego, La Jolla, CA). This vector encodes the human JNK1 kinase and a FLAG tag. The expression vector was transfected into MCF-7/ADR cells and the entire JNK1 coding region was excised from the expression vector. The resulting plasmid was named pCDNA3-JNK1FLAG.

**Cell Culture and Transfections—Drug-resistant human breast carcinoma MCF-7/ADR cells were cultured in McCoy's 5A medium with 10% bovine calf serum (HyClone, Logan, UT) containing 10% fetal bovine serum with 25 mm sodium bicarbonate. Two or three days prior to the experiments, cells were transfected with the HindIII-XbaI fragment of pCMV5-JNK1 containing the entire JNK1 coding region into the expression vector pCDNA3. The resulting plasmid was named pCDNA3-JNK1FLAG.

**Glucose Deprivation Treatment—MCF-7/ADR cells were allowed to grow in a mixture of 95% air and 5% CO₂. Transfections were performed using Lipofectace reagent (Life Technologies, Inc.) per the manufacturer's instructions. McCoy's 5A medium containing 400 µg/ml Genetecin (Life Technologies, Inc.) was placed onto cells 48 h post-transfection to select for transfected cells.

**Glucose Deprivation Treatment—**MCF-7/ADR cells were suspended three times with Hank's balanced salt solution prewarmed at 37 °C. Cells were then treated with glucose-free McCoy's 5A medium with 10% diazoyed bovine calf serum (Life Technologies, Inc.) for the specified time intervals. Assay for c-Jun-associated Kinase Activity—To assay for c-Jun-asso-
c-Jun Phosphorylation Mediated by JNK and Lyn Kinase

Hypoglycemia-induced tyrosine phosphorylation. MCF-7/ADR cells were treated in glucose-free medium for different time intervals as indicated in the figure. An equal amount of protein (30 μg) from the cell lysates was separated by SDS-PAGE and analyzed by Western blotting with anti-phosphotyrosine antibodies.

Fig. 1. Hypoglycemia-induced tyrosine phosphorylation. MCF-7/ADR cells were incubated in glucose-free medium and harvested at various time points. Western blot analysis with anti-phosphotyrosine antibody showed that there was a significant increase in tyrosine phosphorylation of cellular proteins (Fig. 1). Such increase of tyrosine phosphorylation was detectable within 5 min during glucose deprivation treatment to at least 2 h after the treatment. The early increase of tyrosine phosphorylation led us to hypothesize that activation of protein tyrosine kinases is probably one of the upstream signals of the glucose deprivation-induced response in MCF-7/ADR cells.

Activation of Lyn Kinase by Hypoglycemic Treatment of MCF-7/ADR Cells—Because src family kinases have been shown to be involved in a variety of cellular responses, such as to growth factors, oxidative stress, and G protein-coupled signaling cascades (24–26), we tested the possibility that src family tyrosine kinases are involved in the hypoglycemia-induced responses in MCF-7/ADR cells. Several src family members, Abl, Lyn, and Lck were immunoprecipitated from MCF-7/ADR cell lysates after glucose deprivation treatment. Tyrosine kinase assay of the immunoprecipitates showed that although the activities of Abl, Lyn, and Lck were not significantly changed during the 4 h of the glucose deprivation treatment, Lyn kinase was activated 3–4-fold (Fig. 2). The activation of Lyn kinase was obvious within 5 min during the glucose deprivation treatment and lasted for at least 4 h. Compared with the time course of tyrosine phosphorylation in MCF-7/ADR cells under the same conditions, the early activation of Lyn kinase indicates that the activated Lyn kinase is involved in the induction of tyrosine phosphorylation in MCF-7/ADR cells during glucose deprivation treatment.

Hypoglycemia-induced Phosphorylation of c-Jun by JNK1—To investigate the mechanism for the induction of AP-1 activity during glucose deprivation treatment, we assayed for c-Jun kinase activity in the lysates of MCF-7/ADR cells using recombinant GST-c-Jun as the substrate. Fig. 3A showed that there was a significant increase in c-Jun phosphorylation after glucose deprivation treatment. To further confirm that the observed activation of c-Jun kinase activity was due to an increase in JNK1...
activity, we performed the kinase assay using transfected MCF-7/ADR cells that express an epitope (FLAG)-tagged JNK1. We observed that JNK1 immunoprecipitated from the transfected cells after hypoglycemia treatment caused significantly greater phosphorylation of GST-c-Jun than untreated cells, indicating that JNK1 is strongly activated by the glucose deprivation treatment (Fig. 3B). However, when the similar assays were performed using ERK1 and ERK2 immunoprecipitated from glucose deprivation-treated MCF-7/ADR cells, we did not detect any significant phosphorylation of c-Jun by ERKs (Fig. 3C).

**Lyn Antisense Oligonucleotide Inhibited Glucose Deprivation-induced Lyn Kinase and JNK1 Activity**—To study whether the activation of Lyn kinase participated in signaling to the activation of c-Jun, we examined the effect of Lyn antisense oligonucleotide on glucose deprivation-induced c-Jun kinase activity in MCF-7/ADR cells. Fig. 4A showed that compared with treatment with Lyn sense oligonucleotide, Lyn antisense oligonucleotide inhibited over 80% of Lyn kinase activity in cells growing in normal medium and in the glucose-free medium. The JNK kinase assay demonstrated that antisense treatment markedly reduced glucose deprivation-induced JNK1 activity in MCF-7/ADR cells (Fig. 4B). On the other hand, we did not detect any inhibition of glucose deprivation-induced ERK activation with the antisense treatment (Fig. 4C). These results suggest that Lyn kinase is responsible for hypoglycemia-induced JNK1 activation but not ERK activation in MCF-7/ADR cells.

**Hypoglycemia-induced Ras Activation in MCF-7/ADR Cells**—Because it has been demonstrated that Ras can activate the JNK pathway, we examined the extent of Ras activation in MCF-7/ADR cells after hypoglycemia treatment. The activation state of p21ras was measured by metabolic labeling with [32P]orthophosphate and then lysing the cells followed by immunoprecipitation of Ras. TLC assay was performed to determine the amount of GTP and GDP bound to Ras. Fig. 5 showed that there was a significant increase in GTP bound Ras after 5 min of glucose deprivation treatment in MCF-7/ADR cells. Denitometric analysis of the autoradiograph showed that the percentage of active Ras increased approximately 4.5-fold during hypoglycemic treatment.

**DISCUSSION**

The study reported here demonstrated a possible signal transduction pathway in MCF-7/ADR cells that responds to hypoglycemic conditions. Lyn kinase was initially activated by hypoglycemic conditions. Activation of Lyn kinase causes the activation of JNK1, which further phosphorylates and activates c-Jun. Being the major component of the AP-1 transcription factor, c-Jun, once activated, can subsequently activate the transcription of genes, such as bFGF, which contain AP-1 elements (20). The fact that the c-jun gene itself also contains an AP-1 element in its promoter indicates that phosphorylation of c-Jun allows signals in this pathway to be amplified.
phosphorylation of c-Jun activates the transcription of c-jun per se, increasing the amount of available c-Jun for the formation of the AP-1 complex. Second, phosphorylation of c-Jun also increases the activity of the newly formed AP-1 factors, allowing the AP-1 transactivation activity to be further activated.

Although JNK and ERK both belong to the mitogen-activated protein kinase superfamily and are structurally homologous, recent studies have demonstrated that their functions appear to be divergent. JNK, also referred to as stress-activated protein kinase, is a more potent c-Jun kinase than ERK. JNK is primarily induced by stress conditions such as UV radiation (21), hypoxia (27), and osmotic shocks (28). Consistently, we found that a different kind of stress, glucose deprivation, also stimulated JNK1, which is directly responsible for the activation of c-Jun and subsequently the induction of bFGF gene expression. However, data from this and a previous study (29) showed that ERK was also activated by hypoglycemic treatment of MCF-7/ADR cells. The duration of ERK activation was more than 1 h, more persistent than the activation of JNK1, suggesting that hypoglycemia-induced ERK activity probably has some specific function. ERK is responsible for the phosphorylation and activation of the TCF, which is involved in the transcriptional regulation of c-fos. Therefore, it is possible that ERK is responsible for the observed induction of c-fos gene expression induced by hypoglycemia (20). This speculation remains to be tested in future studies.

Lyn kinase, a membrane-associated Src family kinase, has been shown to be involved in a variety of signaling responses, including cheemoattractant receptors (30), radiation (31), and B cell receptor activation (32). In this study, we demonstrated that Lyn kinase activity also can be stimulated by hypoglycemic treatments. Although the downstream substrates of Lyn kinase are largely unknown, recent studies indicate that activated Lyn kinase phosphorylates and associates with the adapter protein Shc through SH2 domain interactions (30). The activated Shc can further associate with the Grb2-Sos complex, leading to the activation of the Ras-dependent pathway (33, 34). Our data from the experiments with Lyn antisense oligonucleotides provided direct evidence that Lyn kinase is responsible for the activation of JNK1. In this study, we observed the activation of Ras in glucose deprivation-treated MCF-7/ADR cells. It is likely that the activation of JNK1 by Lyn kinase is achieved in a Ras-dependent fashion, possibly through the activation of MEKK, the activator of JNK1, as demonstrated by other investigators (35, 36). Therefore, we propose that the signal transduction sequence of Lyn → Ras → MEKK → JNK1 → c-Jun is responsible for the hypoglycemia-induced c-Jun activation in MCF-7/ADR cells.

It remains to be understood how glucose deprivation treatment triggered the activation of Lyn kinase. Preliminary data in our laboratory demonstrated that the activation of Lyn kinase and JNK1 by glucose deprivation treatment of MCF-7/ADR cells can be inhibited by N-acetyl-cysteine, a free radical scavenger, suggesting that free radicals are involved in the glucose deprivation-induced response. Other reports have shown that Src family kinases, including Lyn kinase, also can be activated by ionizing radiation and reactive oxygen (31, 37), which induce cellular free radicals. It is likely that Lyn kinase is activated by a common mechanism mediated by free radicals. Further studies are under way to test this possibility.

REFERENCES

1. Plate, K. H., Breier, G., Millauer, B., Ulrich, A., and Risau, W. (1993) Cancer Res. 53, 5822–5827
2. Millauer, B., Shawyer, L. K., Plate, K. H., Risau, W., and Ullrich, A. (1994) Nature 367, 576–579
3. Witte, L., Fuchs, Z., Haimovitz-Friedman, A., Vlodavsky, I., Goodman, D. W., and Eldar, A. (1999) Cancer Res. 59, 5066–5072
4. Haimovitz-Friedman, A., Vlodavsky, I., Chaudhuri, A., Witte, L., and Fuchs, Z. (1999) Cancer Res. 51, 2552–2558
5. Winkles, J. A., Pedelfy, K. A., and Friesel, R. E. (1992) Cancer Res. 52, 1040–1043
6. Kim, S. J., Glick, A., Sporn, M. B., and Roberts, A. B. (1989) J. Biol. Chem. 264, 1042–1048
7. Kim, S. J., Denhez, F., Kim, K. Y., Holt, J. T., Sporn, M. B., and Roberts, A. B. (1989) J. Biol. Chem. 264, 19373–19378
8. Shibata, P., Baird, A., and Florkiewicz, R. Z. (1991) Growth Factors 4, 2177–2187
9. Angel, P., Hattori, K., Smeal, T., and Karin, M. (1996) Cell 85, 857–865
10. Deng, T., and Karin, M. (1992) Science 255, 62–67
11. Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., and Woodgett, J. R. (1991) Nature 353, 670–674
12. Smeal, T., Bineet, B., Mercola, D. A., Birrer, M., and Karin, M. (1991) Nature 354, 494–496
13. Smeal, T., Bineet, B., Mercola, D., Grover, B. A., Heidecker, G., Rapp, U. R., and Karin, M. (1992) Mol. Cell. Biol. 12, 3507–3513
14. Abel-Hafiz, H. A. M., Heasley, L. E., Kyriakis, J. M., Avruch, J., Kroll, D. J., Johnson, G. L., and Hoeffler, J. P. (1992) Mol. Endocrinol. 6, 2079–2089
15. Gupta, S. S., Campbell, D., Derijard, B., and Davis, R. J. (1995) Science 267, 389–393
16. Treisman, R. (1986) Cell 46, 567–574
17. Treisman, R. (1992) Trends Biochem. Sci. 17, 423–426
18. Gille, H., Sharrocks, A. D., and Shaw, P. E. (1992) Nature 358, 414–417
19. Marais, R., Wynne, J., and Treisman, R. (1995) Cell 73, 381–393
20. Galoforo, S. S., Berns, C., Cote, G., Corry, P. M., and Lee, Y. J. (1996) Mol. Cell. Biochem. 155, 163–171
21. Derijard, B., Hibi, M., Wu, I., Barrett, Y., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) Cell 76, 1025–1037
22. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Avruch, J., and Woodgett, J. R. (1994) Nature 369, 156–160
23. Dean, N. M., and McKay, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11762–11766
24. Gould, K., and Hunter, T. (1988) Mol. Cell. Biol. 8, 3345–3356
25. Mukhopadhyay, D., Tsokas, L., Zhou, X., Foster, D., Brugge, J. S., and Suhatme, V. P. (1995) Nature 377, 577–581
26. Wen, Y., Kurosaki, T., and Huang, Y. X. (1996) Nature 380, 541–544
27. Pembo, C. M., Bonvente, J. V., Avruch, J., Woodgett, J. R., Kyriakis, J. M., and Force, T. (1994) J. Biol. Chem. 269, 26546–26551
28. Galcheva-Gargova, Z., Derijard, B., Wu, I.-H., and Dudis, R. J. (1994) Science 265, 806–808
29. Gupta, A. K., Lee, Y. J., Galoforo, S. S., Berns, C. M., Martinez, A. A., Corry, P. M., Wu, X. Y., and Guan, K. L. (1996) Mol. Cell. Biochem., in press
30. Ptasznik, A., Trayanov-Kaplan, A., and Bolechow, G. M. (1995) J. Biol. Chem. 270, 19869–19873
31. Yang, C., Moroyama, S., Iwabuchi, H., Wang, X., Takata, M., Kurosaki, T., and Yamamura, H. (1995) J. Biochem. (Tokyo) 118, 31–38
32. Myers, D. E., Jun, C., Waddick, K. G., Forsyth, L., Chelstrom, L. M., Gunther, R. L., Tumer, N. E., Bolen, J., and Uckun, F. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9575–9579
33. Li, N., Batzer, A., Daly, R., Vajnik, V., Skolnik, E., Chardin, P., Bar-Sagut, D., Margolis, B., and Schlessinger, J. (1993) Nature 363, 85–88
34. Chardin, P., Cano, J. H., Gale, N. W., Van Aelst, L., Schlessinger, J., Wigler, M. H., and Bar-Sagi, D. (1993) Science 260, 1338–1343
35. Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zon, L. I. (1994) Nature 372, 794–798
36. Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994) Nature 372, 798–800
37. Schieven, G. L., Kirihara, J. M., Myers, D. E., Ledbetter, J. A., and Uckun, F. M. (1993) Blood 82, 1212–1220
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