Volume 295, number 1,2,3, 146–148 FEBS 10533 December 1991

Inhibition of protein tyrosine phosphatase activity by diamide is reversed by epidermal growth factor in fibroblasts

Hugo P. Monteiro*, Yuri Ivaschenko, Roland Fischer and Arnold Stern

Department of Pharmacology, New York University Medical Center, New York, NY 10016, USA

Received 18 September 1991; revised version received 23 October 1991

Diamide (azodicarboxylic acid bis(dimethylamide)) inhibits protein tyrosine phosphatase activity in fibroblasts without altering protein tyrosine kinase activity associated with the epidermal growth factor receptor. The loss of protein tyrosine phosphatase activity caused by diamide is reversed by 2-mercaptoethanol or epidermal growth factor.

EGF receptor; Protein tyrosine phosphatase; Oxidation–reduction

1. INTRODUCTION

Epidermal growth factor (EGF) binds to its cell surface receptor containing tyrosine kinase activity. The activation of the tyrosine kinase activity by the binding process triggers a cascade of events that transduces a signal from the cell surface to the nucleus [1]. Receptor tyrosine kinases catalyse the phosphorylation of tyrosine residues either on their own polypeptide chain or on numerous intrinsic substrates in the signal pathway [2]. Recent evidence suggests that tyrosine kinase phosphorylation could be regulated by a diverse family of enzymes referred to as protein tyrosine phosphatases (PT-Pase) [3,4], whose catalytic activity is dependent on a specific cysteine residue [5]. PTPase activity is found associated with the plasma membrane and/or in the cytosol [6]. Their specific activities are much greater than protein tyrosine kinases and they therefore have the capacity to regulate the level of tyrosine phosphorylation of proteins involved in signal transduction pathways in the cell [7].

2. MATERIALS AND METHODS

EGF from mouse submaxillary glands was obtained from Toyobo Co. Electrophoresis reagents were from Bio-Rad. Diamide and all other chemicals were of reagent grade and were from Sigma. A monoclonal antibody specific for the human EGF receptor (Immunoglobin G 108.1) was provided by Dr. Francoise Bellot (Rorer Central Research, King of Prussia, PA). Affinity-purified antiphosphotyrosine antibodies were a gift of Dr. Ben Margolis (NYU Medical Center, NY). NIH- 3T3 cells expressing the human EGF receptor (HER 14 cells) [8] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, in 100-mm plastic culture dishes. Incubations were done in DMEM containing 20 mM HEPES and 1% bovine serum albumin at 25°C. After incubation, cells were solubilized in lysis buffer A (20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonylfluoride). After 30 min incubation on ice, lysates were spun-down for 10 min at 4°C at 12 000 × g. The supernatants were collected and served as the source of PTPase activity. PTPase activity was assayed by mixing 100 μl of cell lysates with a suspension containing immunoprecipitated [^{32}P]-labeled EGF receptors, prepared essentially as described by Honegger et al. [8]. Reactions were carried out for 30 min at 37°C with stirring. The supernatant was rapidly aspirated and the radiolabeled immunoprecipitates were collected and mixed with 3× concentrated SDS gel-loading buffer and resolved on 7.5% SDS polyacrylamide gel electrophoresis. Immunoblot analysis was performed essentially as previously described [9] except that cells were solubilized in lysis buffer A. Binding experiments were performed according to Lax et al. [9].

Abbreviations: EGF, epidermal growth factor; PTPase, protein tyrosine phosphatase; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate.

**Permanent address:* Fundação Hemocentro de S. Paulo, S. Paulo, Brazil.

Correspondence address: Arnold Stern, Department of Pharmacology, New York University Medical Center, 550 First Avenue, New York, NY 10016, USA. Fax: (1) (212) 263 7133.

3. RESULTS

HER 14 cells were treated with 1 mM diamide and incubated for 30 min at 25°C. PTPase activity was inhibited by 1 mM diamide as shown on SDS-gel electrophoresis (Fig. 1, lane 4). Treatment of cells with 83 nM EGF had no apparent effect on PTPase activity (Fig. 1, lane 3). Pre-incubation of cells with 83 nM EGF followed by incubation with 1 mM diamide (Fig. 1, lane 5) or pre-incubation of cells with 1 mM diamide followed by incubation with 83 nM EGF (Fig. 1, lane 6) showed an increase in PTPase activity when compared to 1 mM diamide (Fig. 1, lane 4), with the increase greatest in the latter situation (Fig. 1, lane 6). The addition of 2-mercaptoethanol to lysates of cells pretreated with 1 mM diamide (Fig. 2, lane 5) resulted in a partial

Published by Elsevier Science Publishers B.V.

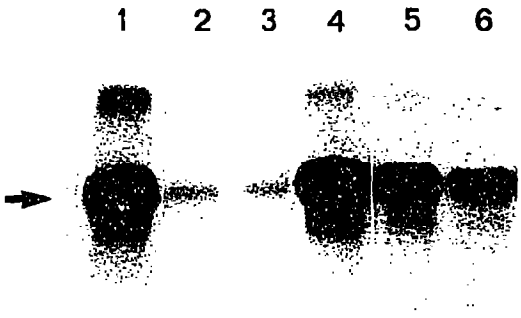

Fig. 1. Effect of diamide on PTPase activity in HER 14 cells. HER 14 cells were incubated for 30 min at 25°C in DMEM containing 20 mM HEPES and 1% bovine serum albumin. PTPase activity was assayed in the supernatants as described in Materials and Methods. (Lane 1) phosphorylated receptor, no lysate added; (lane 2) phosphorylated receptor plus lysate; (lane 3) as 2 with 83 nM EGF; (lane 4) as 2 with 1 mM diamide; (lane 5) as 2 with 83 nM EGF followed by 1 mM diamide added at 5 min; (lane 6) as 2 with 1 mM diamide followed by 83 nM EGF added at 25 min.

reversion of the inhibitory effect of 1 mM diamide (Fig. 2, lane 3) on PTPase activity. Incubation of 1 mM diamide with cell extracts also showed inhibition of PTPase activity (data not shown).

The effect of diamide on autophosphorylation of the EGF-receptor as well as binding of EGF to its receptor was investigated. Diamide at 1 mM did not stimulate autophosphorylation of the EGF-receptor as revealed by immunoblot analysis (Fig. 3, lane 3). The addition of EGF to diamide-treated cells (Fig. 3, lanes 4 and 5) showed tyrosine kinase activity similar to EGF (Fig. 3, lane 2). Furthermore, binding of EGF to its receptor was not impaired by treatment of cells with diamide (data not shown).

4. DISCUSSION

Diamide can inhibit the activity of PTPases which regulate tyrosine kinase activity of the EGF receptor. This inhibition probably occurs by the oxidation of sulf-

Fig. 2. Effect of 2-mercaptoethanol on PTPase activity in HER 14 cells. Conditions and assay were the same as in Fig. 1. (Lane 1) phosphorylated receptor, no lysate added; (lane 2) phosphorylated receptor plus lysate; (lane 3) as 2 with 1 mM diamide; (lane 4) as 2 with 2 mM 2-mercaptoethanol; (lane 5) as 2 with 1 mM diamide plus 2 mM 2- mercaptoethanol.

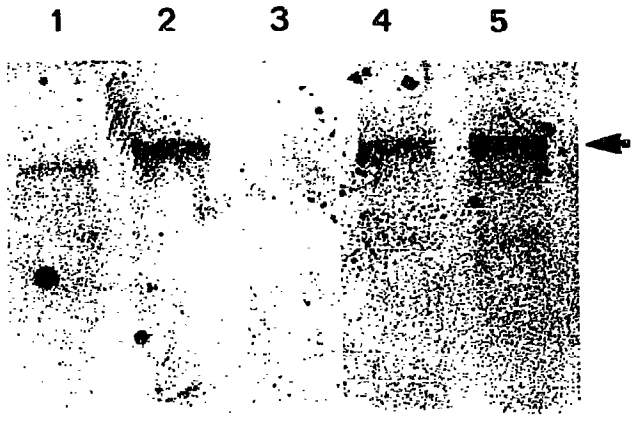

Fig. 3. Effect of diamide on autophosphorylation of the EGF-receptor in HER 14 cells. Conditions were the same as in Fig. 1. Immunoprecipitation of the EGF-receptor by mAb 108 was followed by an immunoblot analysis of the precipitated receptor with anti-phosphotyrosine antibodies. (Lane 1) control; (lane 2) 83 nM EGF; (lane 3) 1 mM diamide; (lane 4) 83 nM EGF followed by 1 mM diamide added at 5 min; (lane 5) 1 mM diamide followed by 83 nM EGF added at 25 min.

hydryl groups on the enzyme and is consistent with the evidence that its activity is dependent on specific cysteine residues in their catalytic domains [5]. The finding that 2-mercaptoethanol reverses the inhibition by diamide provides evidence in favor of oxidized sulfhydryl groups as being responsible for the loss of PTPase activity, and suggests that oxidation–reduction processes may be of importance in the regulation of protein phosphorylation in receptor signal pathways. An oxidation–reduction process was recently demonstrated for maintaining the activity of a protein tyrosine kinase in the endoplasmic reticulum of a B lymphocyte cell line [10].

The attenuation of the diamide-inhibited PTPase activity by EGF indicates that activation of the EGF receptor could have consequences on PTPase activity. The underlying mechanism is not apparent, but EGF must influence processes that regulate the reduction of critical sulfhydryl groups affecting PTPase activity. Consistent with this idea is the observation of increased production of reducing equivalents by EGF activation of the hexose monophosphate shunt in rat kidney cells [11]. That binding of EGF to its receptor was not affected by diamide suggests that the sulfhydryl oxidant modulates PTPase activity with some specificity.

An increase in EGF receptor phosphorylation was not observed in cells treated with diamide and EGF. This indicates that turnover of tyrosine kinase phosphorylation is not a rate-limiting step in the signal transduction pathway of EGF. A similar pattern was observed for the insulin receptor, where phenylarsine oxide, a dithiol complexing agent that inhibits PTPase, did not enhance phosphorylation of the tyrosine kinase activity of the b-subunit of the insulin receptor, but activated tyrosine phosphorylation of a 15 kDa (pp15) protein in adipocytes [12].

Acknowledgements: Supported by National Institutes of Health Grant ES03425. We thank Dr. Joseph Schlessinger for support of Y.I. and R.F. and for careful reading of the manuscript.

REFERENCES

[1] Ullrich, A. and Schlessinger, J. (1990) Cell 61, 203–212.
[2] Margolis, B., Rhee, S.G., Felder, S., Mervic, M., Lyall, R., Levitski, A., Ullrich, A., Silberstein, A. and Schlessinger, J. (1989) Cell 57, 1101–1107.
[3] Lau, K.-H.W., Farley, J.R. and Baylink, D.J. (1989) Biochem. J. 257, 23–36.
[4] Alexander, D.R. (1990) New Biologist 2, 1049–1062.
[5] Tonks, N.K., Diltz, C.D. and Fischer, E.H. (1988) J. Biol. Chem. 263, 6722–6730.
[6] Nelson, R.L. and Branton, P.E. (1984) Mol. Cell Biol. 4, 1003–1016.
[7] Fischer, E.H., Charbonneau, H. and Tonks, N.K. (1991) Science 253, 401–406.
[8] Honegger, A.M., Dull, J.S., Felder, S., van Obberghen, E., Bellot, F., Szapary, D., Schmidt, A., Ullrich, A. and Schlessinger, J. (1987) Cell 51, 199–209.
[9] Lax, I., Johnson, A., Howk, R., Sap, J., Bellot, F., Winkler, M., Ullrich, A., Vennstrom, B., Schlessinger, J. and Givol, D. (1988) Mol. Cell. Biol. 8, 1970–1978.
[10] Bauskin, A.R., Alkalay, I. and Ben-Neriah, Y. (1991) Cell 66, 685–696.
[11] Stanton, R.C. and Seifter, J.L. (1988) Am. J. Physiol. 254, C267–C271.
[12] Bernier, M., Laird, D.M. and Lane, M.D. (1987) Proc. Natl. Acad. Sci. USA 84, 1844–1848.