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Enhancement of epidermal growth factor (EGF) and insulin-stimulated tyrosine phosphorylation of endogenous substrates by sodium selenate

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Sodium selenate stimulated tyrosine phosphorylation of the epidermal growth factor (EGF) receptor in A431 cells and enhanced the tyrosine phosphorylation of endogenous proteins in response to EGF in A431 cells and insulin in NIH 3T3 HIR3.5 cells. These effects occurred without changes in ligand binding, were not abolished by mercaptoethanol in the case of the EGF receptor, and appeared distinct from the effects of vanadate. These results support a role for selenium or selenoproteins in regulating EGF and insulin receptor tyrosine kinase activity and suggest a mechanism whereby selenium-containing compounds contribute to cell growth.

Selenate; Epidermal growth factor (EGF); Insulin; Receptor; Tyrosine phosphorylation

1. INTRODUCTION

Selenium is an essential trace element in humans and animals [1,2] and is required for the activity of glutathione peroxidase [3] which is important for the destruction of reactive oxidative species [4] complementing the antioxidant activity of superoxide dismutase enzymes and vitamin E. Keshan disease is an endemic cardiomyopathy found in Keshan province in China and is causally linked to selenium deficiency [5]. A variety of other selenoproteins have been discovered in mammals [6]. Recently, selenate has been found to be a potent insulin-like agent in isolated rat adipocytes [7] and in vivo [8]. In the present study, we have explored the effects of selenate on EGF receptor phosphorylation and EGF-stimulated phosphorylation in A431 cells, and insulin-stimulated tyrosine phosphorylation in NIH 3T3 HIR3.5 cells [9], cell lines which over-express EGF and insulin receptors, respectively.

2. MATERIALS AND METHODS

Sodium selenate (Na_2SO_4), bovine insulin and sodium orthovanadate (Na_3VO_4) were purchased from Sigma Chemical Company (Poole, Dorset, UK). Nitrocellulose membranes (Schleicher and Schuell) were obtained from Andermann. [^{125}I]EGF and [^{125}I]insulin were purchased from Amersham International (Amersham, Bucks, UK). Rabbit polyclonal anti-phosphotyrosine antibodies were prepared using keyhole-limpet-haemocyanin-conjugated phosphotyramine as immunogen [10] and affinity-purified on phosphotyrosine-agarose (Affigel 15, Bio-Rad). NIH 3T3 HIR3.5 cells [9] were obtained from Dr. J. Whittaker (Stony Brook, NY, USA).

Analysis of tyrosine phosphorylation in A431 cells and NIH 3T3 HIR3.5 cells was performed as described [11] but with some modifications. Cells were routinely cultured in 3.5 cm 6-well dishes (Costar) with DMEM and 10% foetal calf serum, and used when approaching confluence. Approximately 12 h prior to experimental manipulations the medium was replaced with fresh HEPES-buffered DMEM (Sigma) containing 1 mg/ml BSA. Cells were then exposed to sodium selenate and EGF or insulin as indicated. At the end of the incubation, the medium was rapidly aspirated and the cells squirted with 100 μl boiling Luemmli sample buffer containing phosphatase inhibitors [11]. The cell lysates were sonicated in the dish and clarified by centrifugation. The lysates were then loaded onto a 7.5% Tricine-SDS gel [12]. The gels were semi-dry electroblotted for 1 h (Bio-Rad SD Transblot) onto 0.45 μM nitrocellulose which was then blocked for 1 h using 3% BSA in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4) (buffer A). The blocked membranes were probed with anti-phosphotyrosine antibody (0.5–1 μg/ml) in buffer A with 0.05% Triton X-100 for 12 h and washed in TBS, 0.1% Triton X-100, 1 mM EDTA (buffer B) (5 × 100 ml/15 min each) and then probed with anti-rabbit peroxidase conjugate (Sigma) (1:2,000) for 1 h. The membranes were then washed extensively in buffer B as described above. Bound anti-rabbit antibody–peroxidase conjugate was visualised using the luminol-based ECL system (Amersham) as described in the manufacturer's instructions and a permanent record obtained by exposure to preflashed Hyperfilm MP (Amersham), usually for 1–30 s. Band intensities were quantified by densitometric scanning on an LKB Ultroscan XL Enhanced Laser densitometer using LKB 2400 Gelscan XL Version 1.21 software. [^{125}I]EGF binding studies in intact A431 cells were performed essentially as described [13].

The effect of mercaptoethanol on EGF and insulin receptor phosphorylation was examined in Triton X-100 cell lysates. A431 or NIH 3T3 HIR3.5 cells were exposed to selenate and/or EGF/insulin as above and then lysed in ice-cold buffer (50 mM HEPES, pH 7.4, with 10% glycerol, 0.4% Triton X-100, 1 mg/ml bacitracin, 2 mM Na_3VO_4 and 100 mM PMSF). Lysates from selenate-treated cells were treated

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; Na_3VO_4, sodium orthovanadate; Na_2SeO_4, sodium selenate; PMSF, phenylmethylsulfonylfluoride; TBS, Tris-buffered saline.

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with mercaptoethanol before they were combined with lysates from untreated cells. The mixtures were incubated at 37°C for 60 min. Then 3-fold-concentrated Laemlli sample buffer was added, the lysates were boiled and subjected to electrophoresis and immunoblotting as described above.

3. RESULTS

Analysis of phosphotyrosyl-containing proteins in whole cell lysates of A431 cells by immunoblotting showed a M_r 170 kDa protein in control cells (Fig. 1). 0.01 μM EGF stimulated the phosphorylation of this protein by approximately 10-fold (Fig. 1b). These features are consistent with the properties of the EGF receptor. 1 and 10 mM selenate stimulated tyrosine phosphorylation of the EGF receptor by approximately 1.5- and 4-fold, respectively (Fig. 1b). Exposure to lower concentrations of selenate over 5 min did not stimulate EGF receptor phosphorylation (Fig. 1a). When cells were pretreated with 1 and 10 mM selenate for 5 min and then exposed to EGF, selenate- and EGF-stimulated EGF receptor phosphorylation were found to be additive (Fig. 1b). However, a marked potentiation of EGF-stimulated phosphorylation of endogenous substrates was observed after exposure to 10 mM selenate. In particular, 10 mM selenate enhanced the phosphorylation of several endogenous proteins of 80, 84 and 103 kDa by approximately 5-fold.

The effects of selenate were next compared with orthovanadate (Fig. 1b). Although 1 mM vanadate stimulated EGF receptor phosphorylation to the same extent as 1 mM selenate, there was no significant enhancement of endogenous substrate phosphorylation at this maximally effective concentration of vanadate (Fig. 1b).

In intact NIH 3T3 HIR3.5 cells, 1 mM selenate stimulated tyrosine phosphorylation of the insulin receptor β-subunit and a 185 kDa protein (pp185) by approximately 2-fold in the absence of insulin (Fig. 2a). In the presence of insulin, the effect of selenate on the phosphorylation of the insulin receptor β-subunit and pp185 was significantly potentiated (Fig. 2b). In particular, pp185 phosphorylation was enhanced by 3- and 6-fold, respectively, with β-subunit phosphorylation being potentiated over that with insulin alone (Fig. 2b). The conclusion that the 185 kDa protein observed in the present study is pp185 is based on evidence from previous studies [11]. In NIH 3T3 HIR3.5 cells, apart from the insulin receptor β-subunit, insulin rapidly stimulates the tyrosine phosphorylation of an 185 kDa protein with an identical dose–response curve. This 185 kDa protein is rapidly dephosphorylated [11]. These and other features [11] indicate that it is distinct from the insulin receptor and is likely to be insulin receptor substrate-1 (IRS1) [14]. In addition, in NIH 3T3 HIR3.5 cells exposed to selenate, insulin stimulated the tyrosine phosphorylation of another 64 kDa protein (Fig. 2b).

To determine whether the effects of selenate were related to the oxidation of intracellular sulphydryls, EGF receptor phosphorylation in Triton X-100 cell lysates was studied (Fig. 3a). 2-Mercaptoethanol (1 mM) was added to Triton X-100 cell lysates after selenate treatment of intact cells, to assess whether the effects of selenate could be reversed or abolished. 1 mM mercaptoethanol did not diminish selenate-stimulated EGF receptor phosphorylation (Fig. 3a). In contrast, the in-

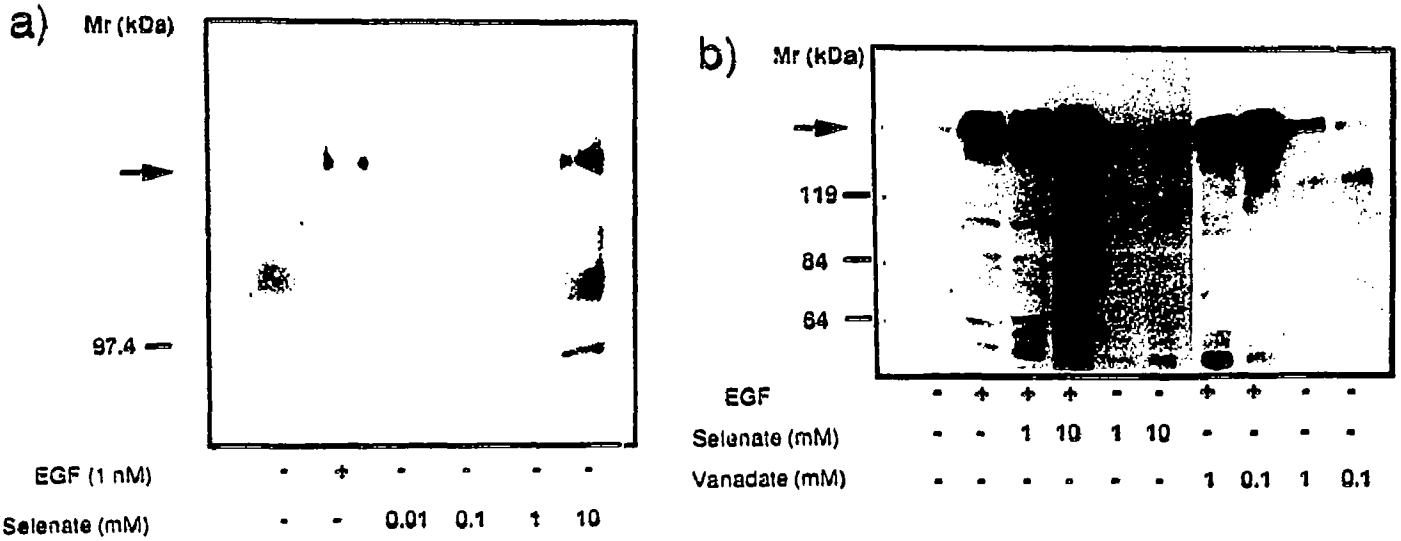

Fig. 1. Effects of selenate on EGF receptor phosphorylation. Autoluminographs of anti-phosphotyrosine immunoblots are shown. (a) Dose–response of selenate-stimulated phosphorylation of the EGF receptor. A431 cells were exposed to sodium selenate (10 μM to 10 mM) then lysed and subjected to electrophoresis and immunoblotting using anti-phosphotyrosine antibodies. (b) Comparison of ligand-stimulated phosphorylation in the presence of selenate and vanadate. A431 cells were exposed to sodium selenate (1 and 10 mM) or vanadate (1 and 0.1 mM) for 5 min and/or EGF (0.01 μM) for 5 min and treated as above. Representative examples of several experiments are shown. The arrows indicate the position of the EGF receptor.

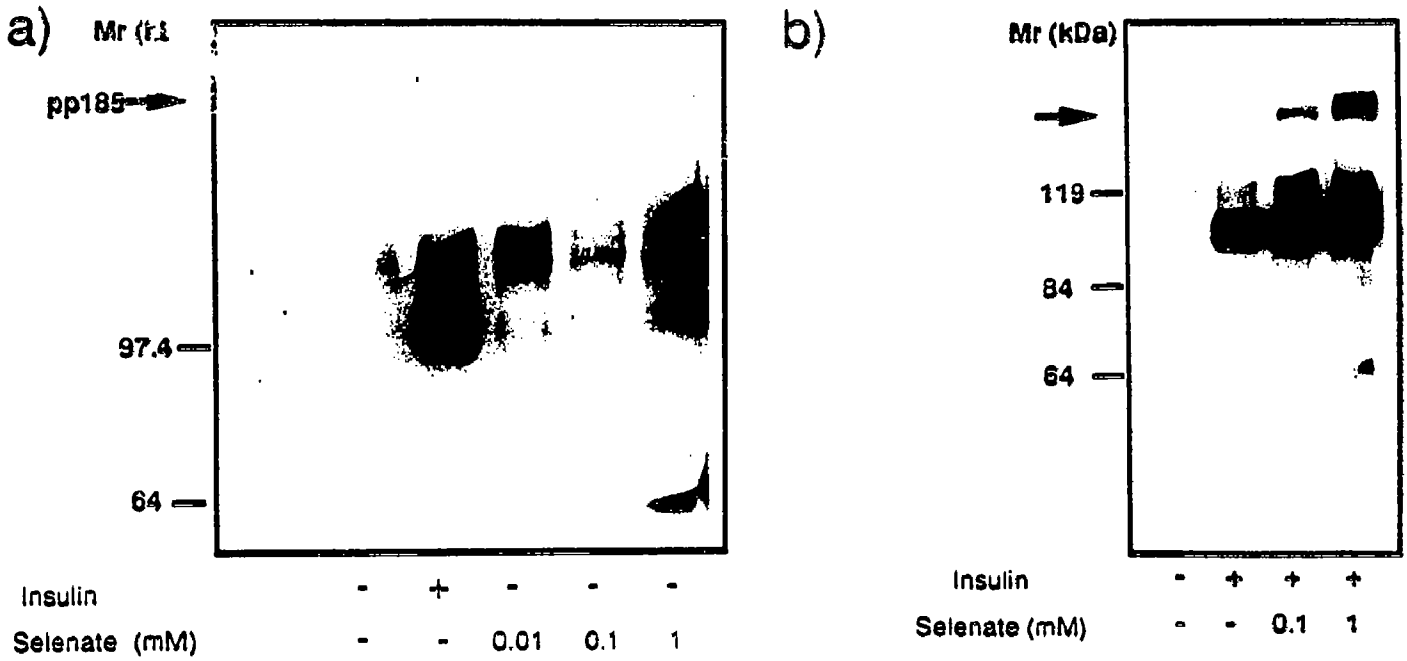

Fig. 2. Tyrosine phosphorylation in NIH 3T3 HIR3.5 cells after selenate treatment. NIH 3T3 HIR3.5 cells were exposed to selenate only (a), or selenate and/or insulin (b) as indicated, and lysed and subjected to electrophoresis and anti-phosphotyrosine immunoblotting as described in section 2. The arrow indicates the position of pp185.

crease in pp185 phosphorylation was largely abolished by the addition of 1 mM mercaptoethanol (Fig. 3b). Neither EGF nor insulin binding was affected by treatment of A431 and NIH 3T3 HIR3.5 cells with selenate (data not shown).

4. DISCUSSION

Selenium is an essential trace element in humans, animals and for the growth of cells in culture [1,2]. Despite its known role in glutathione peroxidase [3] and other selenoproteins [5], the mechanism of action of selenium in regulating cell growth remains to be elucidated. This study provides the first evidence for the regulation of EGF receptor tyrosine phosphorylation and kinase activity by a selenium-containing compound, sodium selenate.

Insulin-like effects of selenate were first observed in rat adipocytes [7] by Ezaki. In isolated rat adipocytes, selenate has several insulin-like effects, including stimulating tyrosine phosphorylation of numerous endogenous cellular proteins, glucose transport, cAMP phosphodiesterase activity, and ribosomal S6 phosphorylation [7]. Subsequent experiments in streptozotocin-induced diabetic rats have confirmed these insulin-like effects on glucose uptake [8].

We embarked on this study to gain further insight into the cell biology of these growth factor-like effects of selenate using cell lines that over-express EGF and insulin receptors. The high levels of expression facilitate the rapid analysis of ligand-stimulated endogenous substrate phosphorylation by anti-phosphotyrosine immunoblotting. In A431 cells, selenate stimulates EGF receptor phosphorylation and enhances its endogenous tyrosine kinase activity as is apparent from its effects on substrate phosphorylation. Orthovanadate, a well-studied phosphotyrosine phosphatase inhibitor, was less effective than selenate at increasing EGF receptors and enhancing EGF-stimulated tyrosine phosphorylation of endogenous substrates for the EGF receptor. This indicates that the effects of selenate are unlikely to be due to the inhibition of phosphotyrosine phosphatases, which is consistent with the evidence from rat adipocytes where selenate did not inhibit the activity of phosphotyrosine phosphatases in cell lysates [7]. In NIH 3T3 HIR3.5 cells, selenate increases the insulin-stimulated phosphorylation of a 185 kDa protein. Insulin receptor substrate-1 (IRS1) [14] is a ubiquitous 185 kDa substrate that displays properties of being a major insulin receptor substrate involved in insulin action and may function as a multi-site docking protein during insulin-initiated signal transduction. The tyrosine phosphorylated pp185 band observed in immunoblots from NIH 3T3 HIR3.5 cells in previous studies and the present study probably represents murine IRS-1 in whole or part [11]. The kinetics of pp185 or IRS1 phosphorylation indicate a relatively early and proximal role in insulin-initiated signal transduction [14]. The present study has provided evidence that selenate regulates this proximal component of the insulin-initiated signal

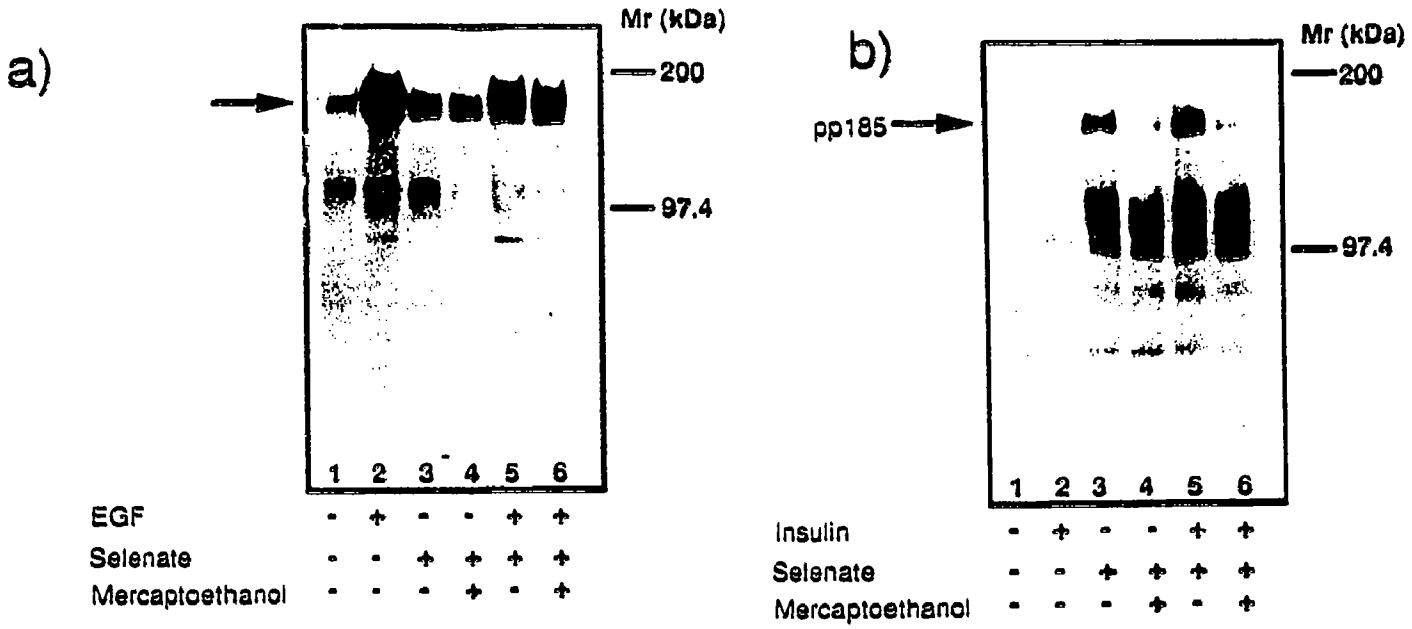

Fig. 3. Effects of mercaptoethanol on selenate-stimulated phosphorylation in Triton X-100 lysates of A431 (a) and NIH 3T3 HIR3.5 cells (b). A431 or NIH 3T3 HIR3.5 cells were incubated in DMEM and then exposed to 10 mM selenate for 5 min and EGF (0.01 μM) or insulin (0.1 μM), respectively, for 5 min. The cells were lysed in 0.4% Triton X-100. Lysates from control cells (lanes 1 and 2) were combined with lysates from selenate-treated cells (lanes 3-6) of which two aliquots (lanes 4 and 6) had been treated with 1 mM mercaptoethanol. All the lysates were then incubated at 37°C for 60 min. The lysates were subjected to SDS-PAGE, anti-phosphotyrosine immunoblotting and autoluminography as described. A typical example of several experiments is shown. In (a) the arrow indicates the EGF receptor.

transduction pathway, substantiating the initial observations of Ezaki [7], as well as identifying a possible site of action. This effect may be sufficient to explain the effect of selenate on more distal components of the signal transduction pathway although it does not exclude direct distal effects as well. The mechanism of action of selenate in these situations is not clear, but could be related to the oxidation of critical SH groups in susceptible regulatory proteins as selenate oxidises SH groups in vitro [15]. The lack of effect on ligand binding indicates that the effects of selenate are probably mediated intracellularly. Phosphotyrosine phosphatases regulate growth factor receptor tyrosine kinase activity by promoting dephosphorylation [16,17]. Critical cysteine residues in these phosphatases regulate their activity [18,19]. Consequently, the effects of selenate may be explained by this mechanism. If selenate acts by inhibiting phosphotyrosine phosphatases, then why do its effects on the EGF receptor appear distinct from vanadate? It is possible that only selected phosphotyrosine phosphatases are affected by selenate. Mercaptoethanol failed to reverse the effects of selenate on EGF-stimulated phosphorylation suggesting that sulphydryl group oxidation may not be the only mechanism in the action of selenate on EGF-stimulated phosphorylation, but appears to be important in insulin-stimulated phosphorylation. Furthermore, not all selenate-induced modifications may not be susceptible to reduction by mercaptoethanol.

The results of this study illustrate several differences and similarities in the regulation of EGF receptor and insulin receptor tyrosine phosphorylation in the cell lines used. Firstly, 1 mM selenate appears less effective at stimulating insulin receptor phosphorylation than EGF receptor phosphorylation. In rat adipocytes, selenate did not stimulate insulin receptor phosphorylation, but clearly enhanced insulin-stimulated kinase activity towards endogenous substrates [7]. The potentiation of insulin-stimulated insulin receptor β-subunit phosphorylation is likely to be a reflection of this enhanced endogenous tyrosine kinase activity in the present study. Selenate potentiates the ligand-stimulated phosphorylation of endogenous substrates for both the EGF and insulin receptors. Mercaptoethanol was more effective at reversing the effects of selenate on insulin-stimulated phosphorylation receptor than EGF-stimulated phosphorylation. An explanation for this difference is not readily apparent but may be explained by structural differences in the nature of individual phosphotyrosine phosphatases that regulate dephosphorylation of different tyrosine kinase receptors and which may dictate their susceptibility to oxidative-reductive modification.

Although selenium is a trace element, these effects of selenate are only observed in the presence of pharmacological concentrations. However, the precise mechanisms by which toxic levels of selenium interfere with cellular function are poorly understood and consequently this study may provide a basis for understand-

ing selenium toxicity in humans and animals [20]. The effects of selenate on A431 cells, NIH 3T3 HIR3.5 cells and rat adipocytes [7] have similar dose–response curves in terms of the maximally effective concentrations. Hence, selenate may prove useful in the identification of novel EGF and insulin receptor substrates owing to its effects on ligand-stimulated tyrosine phosphorylation of endogenous substrates. A goal of future studies would be further characterization of these substrates.

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