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The insulinomimetic agents H_2O_2 and vanadate stimulate tyrosine phosphorylation of potential target proteins for the insulin receptor kinase in intact cells

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H_2O_2 and vanadate are known insulinomimetic agents. Together they induce insulin's bioeffects with a potency which exceeds that seen with insulin, vanadate or H_2O_2 alone. We have previously shown that a combination of H_2O_2 and vanadate, when added to intact cells, rapidly stimulates protein tyrosine phosphorylation, owing to the inhibitory effects of these agents on intracellular protein tyrosine phosphatases (PTPases). Employing Western blotting with anti-phosphotyrosine antibodies, we have now identified in Chinese-hamster ovary (CHO) cells transfected with a wild-type insulin-receptor gene (CHO.T cells) several proteins (e.g. pp180, 125, 100, 60 and 52) whose phosphotyrosine content is rapidly increased upon treatment of the cells with a combination of insulin and 3 mM-H_2O_2. Tyrosine phosphorylation of these and additional proteins was further potentiated when 100 μM-sodium orthovanadate was added together with H_2O_2. The effects of insulin, insulin/H_2O_2, and H_2O_2/vanadate on tyrosine phosphorylation were markedly decreased in CHO cells transfected with an insulin-receptor gene where the twin tyrosines 1162 and 1163 were replaced with phenylalanine (CHO.YF-3 cells). Similarly, most of these proteins failed to undergo enhanced tyrosine phosphorylation in parental CHO cells incubated in the presence of insulin or the insulinomimetic agents. Our findings suggest that inhibition of PTPase activity by H_2O_2/vanadate augments the autophosphorylation of tyrosines 1162 and 1163 of the insulin receptor kinase, leading to its activation in an insulin-independent manner. As a result, tyrosine phosphorylation of potential targets for this enzyme takes place. Failure of H_2O_2/vanadate to induce phosphorylation of these proteins in receptor mutants lacking these twin tyrosine residues supports this hypothesis.

INTRODUCTION

H_2O_2 and vanadate mimic several of the metabolic and growth-promoting effects of insulin and related growth factors. Vanadate stimulates glucose uptake and oxidation [1,2], activates glycogen synthase [3], induces the translocation of receptors for insulin-like growth factor II to the plasma membrane [4], and down-regulates the cell-surface receptors for insulin and growth hormone [5]. Vanadate also induces cell growth [6] and transformation [7]. H_2O_2, like vanadate, mimics several of insulin's actions. It enhances glucose transport and oxidation [8,9], inhibits hormone-stimulated lipolysis [10], stimulates lipogenesis [9], activates pyruvate dehydrogenase [9], and stimulates glycogen synthase [11]. Incubation of intact adipocytes [12,13] or H-35 rat hepatoma cells with H_2O_2 [14] enhances [12,13] phosphorylation of the insulin receptor and activates the insulin receptor kinase (IRK). When added together, H_2O_2 and vanadate act with a potency much greater than the sum of their individual effects [12,15]. They cause a marked increase in the binding of insulin-like growth factor II to adipocytes [12] and stimulate IRK activity several-fold [15,16]. These synergistic effects of H_2O_2 and vanadate exceed those seen with insulin, vanadate or H_2O_2 alone [12,15,16].

Activation of the insulin receptor [17,18] and other protein tyrosine kinases [19,20] leads to enhanced tyrosine phosphorylation of endogenous cellular proteins. The insulin-like effects of H_2O_2 and vanadate could therefore be mediated, at least in part, by tyrosine phosphorylation of intracellular proteins. Indeed, we have shown [21] that preincubation of Fao cells with H_2O_2 potentiates insulin-dependent tyrosine phosphorylation of pp180, a putative substrate of the IRK [22], as well as of additional proteins (pp150, 114, 100, 85, 68, 56) which are otherwise undetected in cells treated with insulin alone. We have further demonstrated [23–26] that a combination of vanadate and H_2O_2 markedly enhances protein tyrosine phosphorylation in Fao and other cell types, and the proteins phosphorylated in response to the combined action of H_2O_2 and insulin are also phosphorylated in response to treatment with H_2O_2 and vanadate [23]. The potentiating effects of H_2O_2 and vanadate on protein tyrosine phosphorylation are rapid, specific, and reversible, and could be attributed to the inhibitory effects of these agents [23] on activities of intracellular protein tyrosine phosphatases (PTPases) [27,28]. However, since this inhibition is exerted on PTPases with relaxed specificity, we were interested to find why potential targets of the IRK are preferentially detected in cells treated with these insulinomimetic agents.

Our working hypothesis predicts that H_2O_2 and vanadate augment protein tyrosine phosphorylation in two ways. They inhibit dephosphorylation of substrate proteins, but at the same time they inhibit the dephosphorylation of protein tyrosine kinases. Since autophosphorylation of IRK enhances its activity [17,18], H_2O_2/vanadate could induce its activation, as they promote the accumulation of tyrosine-phosphorylated forms of this enzyme [16,23]. Accordingly, substrates for IRK are likely to represent at least some of the tyrosine-phosphorylated proteins observed in H_2O_2/vanadate-treated cells.

To address this possibility, we compared the effects of insulin, H_2O_2, and H_2O_2/vanadate on protein tyrosine phosphorylation in Chinese-hamster ovary (CHO) cells transfected with a wild-type insulin-receptor gene (CHO.T cells) [29] and in CHO cells

Abbreviations used: PTPases, protein tyrosine phosphatases; CHO, Chinese-hamster ovary; IRK, insulin receptor kinase.
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transfected with an insulin-receptor gene where the twin tyrosines 1162 and 1163 were replaced with phenylalanine (CHO.YF-3 cells) [29]. These cells manifest a substantial decrease in autophosphorylation of IRK *in vivo* and a parallel decrease in insulin-activated uptake of 2-deoxyglucose [29].

We could demonstrate that several proteins undergo enhanced tyrosine phosphorylation in CHO.T cells incubated with insulin/H_2O_2 or H_2O_2/vanadate, whereas phosphorylation of these proteins is markedly decreased in CHO.YF-3 or parental CHO cells. The significance of these findings, and the potential use of H_2O_2/vanadate as tools to purify target proteins for IRK, are discussed.

EXPERIMENTAL

Materials

Anti-phosphotyrosine antibodies were prepared as previously described [21]. Mono[^{125}I]iodo-labelled (at tyrosine-14 of the A-chain) human insulin (2000 Ci/mmol) was obtained from Amersham (U.K.). Pig insulin was from Elanco, Indianapolis, IN, U.S.A. Gel reagents were from Bio-Rad.

Cell cultures

CHO cells transfected either with a wild-type human insulin-receptor gene (CHO.T) or with a mutated insulin-receptor gene, where tyrosine-1162 and -1163 were replaced by phenylalanine (CHO.YF-3), were grown in F-12 medium supplemented with 10% (v/v) fetal-calf serum as previously described [29].

^{125}I-Insulin-binding assay

The assay was performed as described in [30]. The number of receptors/cell was calculated from the Scatchard analysis of the binding data.

Ligand treatment of intact cells

Cells, grown in 60 mm-diam. plates, were deprived of serum for 16 h before each experiment. At 1 h before treatment, the medium was replaced with fresh medium supplemented with 1 mg of BSA (Sigma, RIA grade)/ml. Insulin, H_2O_2, sodium orthovanadate or their combination were added to the medium as indicated. After incubation, cells were washed twice with ice-cold phosphate-buffered saline and frozen in liquid nitrogen. Cell extracts were prepared by addition of buffer I (Hepes 50 mM, pH 7.6, NaCl 150 mM, Triton X-100 1%, SDS 0.1%, NaF 50 mM, sodium pyrophosphate 10 mM, sodium orthovanadate 2 mM, EDTA 10 mM, EGTA 2 mM, phenylmethanesulphonyl fluoride 1 mM, aprotinin 10 μg/ml, and leupeptin 5 μg/ml). The extracts were centrifuged for 30 min at 4 °C at 12000 *g*. Supernatants were mixed with (5×) concentrated Laemmli sample buffer [31] containing 20 mM-dithiothreitol, resolved by SDS/PAGE (7.5% gels) under reducing conditions and transferred to nitrocellulose papers for immunoblotting.

Western blotting with anti-phosphotyrosine antibodies

Electrophoretic transfer from the gels to the nitrocellulose papers, incubation with anti-phosphotyrosine antibodies, and decoration with ^{125}I-labelled goat anti-rabbit antibodies were carried out as previously described [21].

RESULTS

Effect of insulin on protein tyrosine phosphorylation in CHO.T and CHO.YF-3 cells

Immunoblotting of cell extracts with anti-phosphotyrosine antibodies was applied in order to identify potential protein substrates for the IRK in intact CHO.T and CHO.YF-3 cells. Consistent with previous studies [32], we could demonstrate (Fig. 1) that incubation of CHO.T cells with insulin resulted in enhanced tyrosine phosphorylation of two proteins with molecular masses of 180 and 95 kDa (pp180 and pp95) (Fig. 1*a*). The former is a potential substrate of IRK, known as IRS-1 [33], whereas the latter most likely corresponds to the β subunit of the receptor itself. Phosphorylation of pp180 was detected 30 s after insulin treatment, reached a maximum by 5 min and then declined to basal levels by 30 min. By contrast, no phosphorylation of pp180 or of pp95 could be detected under our assay conditions in CHO.YF-3 cells, which bear kinase-defective receptors. The lack of phosphorylation of pp180 was evident in cells stimulated with 10 pM–0.1 μM-insulin (Fig. 1*b*). The comparison between the cell lines was carried out by normalizing the cell extracts per equal amounts of protein. This was a valid comparison, since the two cell lines expressed approximately the same amount of insulin receptors per cell (8×10^5 and 5×10^5 in CHO.T and CHO.YF-3 respectively).

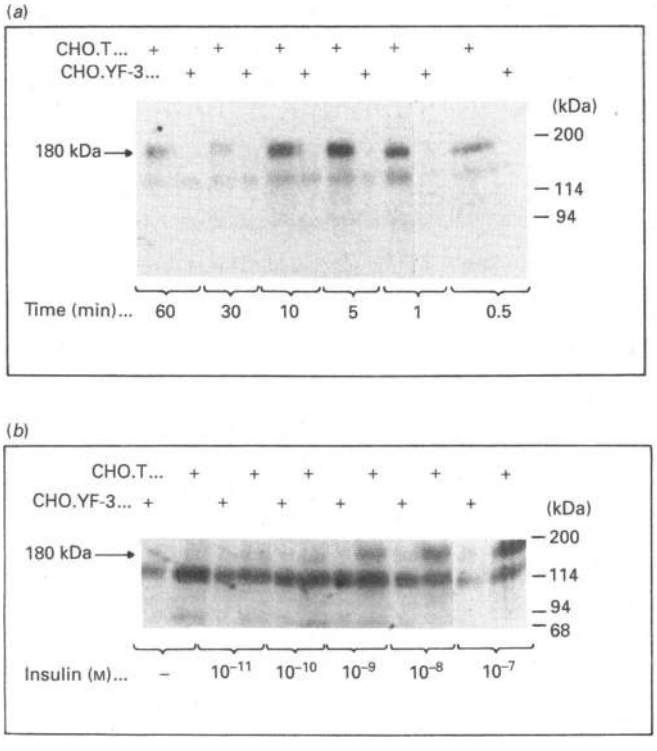

Fig. 1. Effect of insulin on protein tyrosine phosphorylation in CHO.T and CHO.YF-3 cells

Confluent monolayers of CHO.T and CHO.YF-3 cells were deprived of serum for 16 h before treatment. The medium was then replaced with fresh medium containing 1 mg of BSA (RIA grade)/ml. The cells were incubated without or with 0.1 μM-insulin for increasing time periods (*a*) or with increasing concentrations of insulin (10 pM–0.1 μM) for 5 min (*b*). The cells were washed immediately after treatment with ice-cold phosphate-buffered saline and frozen on liquid nitrogen. The 300 μl of extraction buffer (see the Experimental section) was added to the plates, and the cells were scraped off with a rubber policeman, and centrifuged at 12000 *g* for 30 min at 4 °C. Samples (80 μl) of the supernatants, yielding about 100 μg of protein, were resolved by SDS/PAGE on 7.5% gels and blotted with affinity-purified anti-phosphotyrosine antibodies. This is a representative of two identical experiments with essentially the same results.

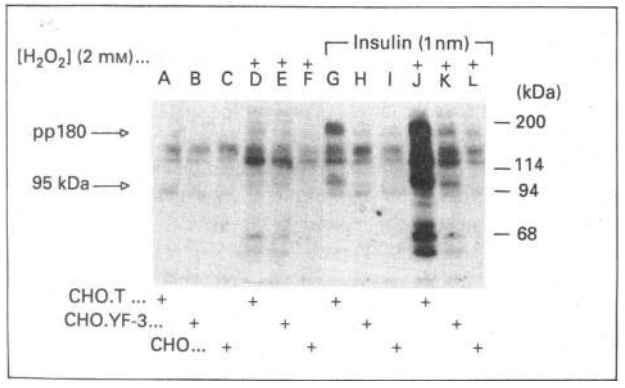

Fig. 2. Effect of H_2O_2 and insulin on protein tyrosine phosphorylation in CHO cells

CHO.T, CHO.YF-3 and parental CHO cells were incubated for 20 min with buffer (A–C, G–I) or 2 mM-H_2O_2 (D–F, J–L). Insulin (1 nM) (G–L) or buffer (A–F) was added for the last 5 min of the incubation period. The rest of the experiment was performed as described in the legend for Fig. 1. This is one out of two experiments that yielded essentially identical results.

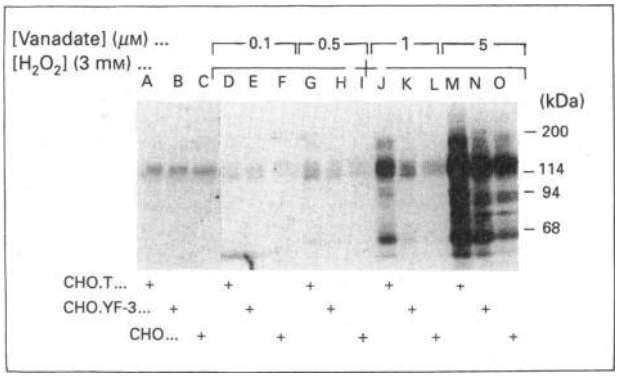

Fig. 3. Effect of H_2O_2 and vanadate on protein tyrosine phosphorylation in CHO cells

CHO.T, CHO.YF-3 and parental CHO cells were incubated for 20 min with buffer (A–C) or 3 mM-H_2O_2 (D–O) and the indicated concentrations of vanadate. The cells were then solubilized and the samples were further processed as described in the legend to Fig. 1. This is one out of two experiments that yielded essentially identical results.

Effects of H_2O_2 on insulin-dependent protein tyrosine phosphorylation in CHO cells

Incubation of CHO cells with the insulinomimetic agent H_2O_2 (Fig. 2, lanes D–F) had only small effects on protein tyrosine phosphorylation. Enhancement of a single major protein with molecular mass of 125 kDa was evident in the CHO.T cells (lane D), whereas less intensive phosphorylation of this protein occurred in CHO.YF-3 cells (lane E). When insulin was added to H_2O_2-pretreated CHO.T cells (lane J), we observed a marked increase, of at least 5-fold, in tyrosine phosphorylation of pp180, and of additional proteins (pp125, 115, 95, 85, 68 and 60). This was in sharp contrast with the trivial effects of insulin on the phosphorylation of pp180 and other proteins in H_2O_2-pretreated CHO.YF-3 cells (lane K). Similarly, no intense phosphorylation of pp95, pp180 or other potential targets could be detected in parental non-transfected CHO cells, whether they were incubated in the presence of insulin (lane I), H_2O_2 (lane F) or their combination (lane L). Phosphorylations stimulated by H_2O_2 and insulin occurred exclusively on tyrosine residues, as immunoblotting with anti-phosphotyrosine antibodies could be completely inhibited by 10 μM-phosphotyrosine, but not by 250 μM-phosphoserine or phosphothreonine (results not shown). The potentiating effects of H_2O_2 on insulin-mediated tyrosine phosphorylation were time-dependent. Half and maximal effects were obtained when CHO.T cells were treated with H_2O_2 for 10 and 30 min respectively (results not shown). We have previously demonstrated [21] that insulin and H_2O_2 enhanced protein tyrosine phosphorylations only when added to intact cells. Addition of H_2O_2 to extracts of untreated cells (during the time of solubilization) in the presence or absence of insulin failed to enhance any protein tyrosine phosphorylation.

Effects of H_2O_2 and vanadate on protein tyrosine phosphorylation

Consistent with our previous observations in other cell types [23–26], incubation of each of the three CHO cell lines for 30 min with 10 μM–1.0 mM-sodium orthovanadate failed to stimulate protein tyrosine phosphorylation (results not shown). These results are at variance with a previous report [32], where 0.5 mM-vanadate augmented protein tyrosine phosphorylation in CHO.T cells, but the reason for these differences are currently unknown. In contrast, when CHO.T cells were incubated in the presence of

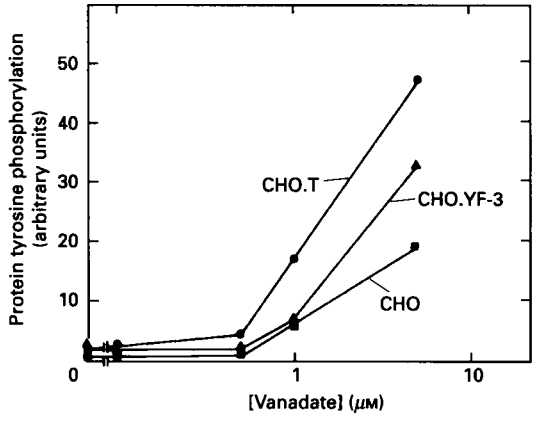

Fig. 4. Quantitative analysis of the effects of H_2O_2 (3 mM)/vanadate on protein tyrosine phosphorylation

The autoradiogram presented in Fig. 3 underwent densitometry, and the intensity of the indicated phosphorylated proteins was plotted in arbitrary units, as a function of the increasing concentrations of vanadate.

3 mM-H_2O_2 and increasing concentrations of vanadate, we observed a dose-dependent elevation in protein tyrosine phosphorylation (Fig. 3). No measurable effect could be detected in CHO.T cells incubated with 3 mM-H_2O_2 and up to 0.5 μM-vanadate, but at 3 mM-H_2O_2/1 μM-vanadate we observed an increase in protein tyrosine phosphorylation in the CHO.T cells, with no comparable increase in CHO.YF-3 or parental CHO cells (Fig. 3, cf. lane J with K or L). A further increase in the vanadate concentrations augmentated protein tyrosine phosphorylation of a similar set of proteins in both CHO.T and CHO.YF-3 cells, with the greatest effect in the CHO.T cells (Fig. 3, lanes M and N). Only one protein, pp125, underwent a comparable tyrosine phosphorylation in all three CHO cell lines (lanes J, K and L), whereas phosphorylation of pp60 occurred to the same extent in both CHO.T and CHO.YF-3 cells, but not in the parental CHO cells. Quantitative analysis of the results revealed (Fig. 4) that the differences in the level of protein tyrosine phosphorylation diminished with increasing concentrations of vanadate. Maximal response (at 5 μM-vanadate) of CHO.T and CHO.YF-3 cells was only 2.4- and 1.7-fold greater

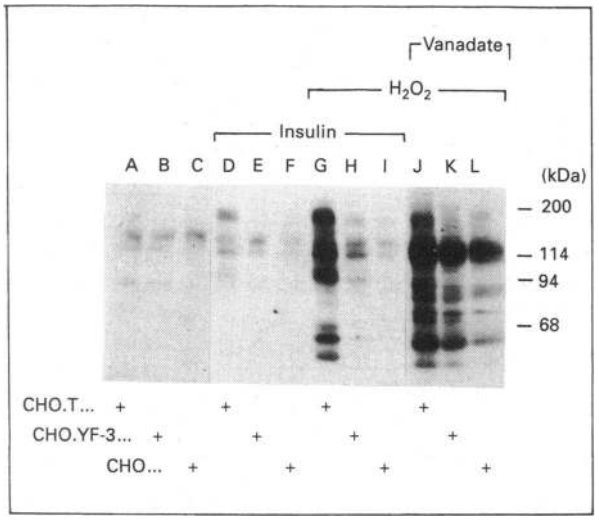

Fig. 5. Comparison of the effects of insulin, H_2O_2/insulin and H_2O_2/vanadate on protein tyrosine phosphorylation

The indicated CHO cell lines were incubated for 20 min with buffer (A–F), 3 mM-H_2O_2 (G–I) or 3 mM-H_2O_2 + 5 μM-vanadate (J–L); 10 nM-insulin (D–I) or buffer (A–C, J–L) was added for the last 5 min of the incubation period. The cells were washed immediately after treatment with ice-cold phosphate-buffered saline and frozen on liquid nitrogen. Processing of the samples was carried out as described in the legend to Fig. 1.

than that of parental CHO cells, respectively. This could be accounted for by the fact that at high concentration H_2O_2/vanadate fully inhibited PTPase activity. The total inhibition of the dephosphorylation reactions, together with the intrinsic activity of the endogenous IRK and related tyrosine kinases, could be sufficient to induce significant accumulation of tyrosine-phosphorylated proteins.

Comparison between the effects of H_2O_2/insulin and H_2O_2/vanadate on protein tyrosine phosphorylation

Fig. 5 presents a comparison between the proteins phosphorylated in response to insulin (and H_2O_2) and those phosphorylated after treatment with H_2O_2/vanadate. H_2O_2/vanadate induced in CHO.T cells (Fig. 5, lane J) tyrosine phosphorylation of a similar set of proteins to those detected in cells incubated in the presence of H_2O_2/insulin (Fig. 5, lane G, and Fig. 2). Most prominent are proteins with molecular masses of 180, 125, 115, 94, 68 and 60 kDa. As shown above (Figs. 2 and 3), phosphorylation of these proteins is markedly decreased in CHO.YF-3 cells, and it is even further decreased in parental CHO cells incubated under similar conditions.

DISCUSSION

We have previously shown that pretreatment of rat hepatoma Fao cells with H_2O_2 augments the effect of insulin on tyrosine phosphorylation of pp180, and enables the detection of several additional putative substrates for the IRK [21]. We have further demonstrated [23] that H_2O_2 acts as an intracellular inhibitor of PTPases and that its effects are augmented in the presence of vanadate. The stimulation of tyrosine kinases [12–14] and the inhibition of PTPase activities [23] mediated by H_2O_2 alone or together with vanadate is presumably the basis of the marked stimulation of protein tyrosine phosphorylation observed in cells treated with a combination of H_2O_2 and insulin or H_2O_2 and vanadate [23–26]. In the present study we provide evidence that at least some of the proteins whose phosphorylation is augmented in such a manner (i.e. pp180, 125, 115, 68, 60) are potential targets of the IRK. This conclusion is based on the fact that phosphorylation of these proteins is markedly stimulated in CHO.T cells, transfected with a wild-type insulin-receptor gene, whereas it occurs to a much lower extent in CHO.YF-3 cells transfected with a kinase-defective mutant [29], or in parental CHO cells. Hence, if we assume that the only difference between the three cell lines is the presence of an excess of functional insulin receptors in CHO.T cells, then the phosphorylation of these proteins is likely to be mediated, directly or indirectly, by IRK.

The capability of H_2O_2 to potentiate phosphorylation of several target proteins for IRK (Fig. 2) that are not readily detected in cells treated with insulin alone (Fig. 1) is not unexpected, in view of the fact that H_2O_2 effectively inhibits intracellular PTPase activities [23]. These enzymes have been implicated in maintaining the intracellular phosphotyrosine content at extremely low levels [27,28,34], a notion supported by the fact that hormones and growth factors, which activate receptor tyrosine kinases, induce a rapid but transient increase in the phosphotyrosine content of their protein substrates. An example is pp180, which *in vivo* undergoes immediate but transient insulin-induced tyrosine phosphorylation (cf. Fig. 1). Thus detection of additional potential substrates for IRK is largely facilitated once intracellular PTPase activities are inhibited by pretreating the cells with H_2O_2 before their stimulation with insulin. The presence of multiple putative targets for IRK is further supported by the fact that several proteins in different cell types {(pp240 [35], 180 [22,33,35], 120 [36], 46 [37], 15 [38], calmodulin [39], microtubule-associated protein-2 (MAP-2) protein kinase [40] and specific serine kinase(s) [41,42]} have been implicated as potential substrates of IRK.

The intriguing observation is the similarity between the proteins phosphorylated in response to H_2O_2 and insulin and those phosphorylated in response to H_2O_2 and vanadate (Fig. 5). It raises the question why a combination of rather non-specific inhibitors of PTPases act similarly to a hormone like insulin with a well-defined specificity. Although we can only speculate, the data accumulated so far ([4,16,23–26] and the present work) seem to indicate that the repertoire of the tyrosine-phosphorylated proteins in H_2O_2/vanadate-treated cells depends on the concentration of these agents and the basal activity of the protein tyrosine kinases that are predominant in the cells under study. Since inhibition of intracellular PTPases is expected to enhance indirectly the activity of protein tyrosine kinases that are stimulated through autophosphorylation, tyrosine kinases such as the insulin and insulin-like growth factor-I receptors are expected to maintain a high basal activity in H_2O_2/vanadate-treated cells, even in the absence of a ligand. Hence, when these agents are added at low concentrations (i.e. 1 μM) to CHO.T cells expressing a large excess of functional IRKs, they mainly facilitate tyrosine phosphorylation of potential substrates for this enzyme. When the concentration of vanadate is increased and the inhibition of PTPase activity is more pronounced, the impaired, albeit significant, basal activity of IRK in CHO.YF-3 cells [26] is sufficient to drive the phosphorylation of its substrate proteins. In view of the above, we cannot rule out the possibility that at least some of the phosphorylated proteins represent substrates for other protein tyrosine kinases that are activated through autophosphorylation. For example, pp125 might represent such a substrate, as its phosphorylation is increased in the parental CHO cells treated with H_2O_2/vanadate. It should also be noted that, although certain proteins like pp180 [43] or phospholipase C-γ [44] are phosphorylated specifically by the insulin/insulin-like growth factor-I receptors, or by the epidermal growth factor/platelet-derived growth factor receptors, respectively, other proteins, such as phosphatidylinositol

3-kinase, are activated by several protein tyrosine kinases, including the insulin receptor [45,46], the platelet-derived growth factor receptors [47], and the oncogene product *v-src* [48]. These findings correlate with the observation that insulin and other growth factors (e.g. platelet-derived growth factor) induce certain overlapping biological activities [49,50]. Thus the proteins phosphorylated in response to H_2O_2/vanadate could serve as substrates to both IRK and related protein kinases.

Our model also predicts that in other cell types, expressing a high basal activity of a different protein tyrosine kinase, treatment with H_2O_2/vanadate will facilitate detection of substrate proteins for that particular kinase. Indeed, we have recently shown [25] high levels of tyrosine-phosphorylated proteins in H_2O_2/vanadate-treated chick lens epithelial cells that were transfected with temperature-sensitive mutant of Rous sarcoma virus and cultured at the permissive temperature (37 °C). Phosphorylation of these proteins was markedly decreased on treatment of these cells with H_2O_2/vanadate at the restrictive temperature (42 °C).

The marked potentiation of protein tyrosine phosphorylation in response to H_2O_2/vanadate could be utilized in order to isolate large amounts of substrate proteins from organ tissues. In preliminary studies we could demonstrate enhanced tyrosine phosphorylation of a similar set of proteins in rat livers injected with a combination of H_2O_2/vanadate. Attempts to purify these proteins are now feasible.

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