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H_2O_2-Induced Phosphorylation of ERK1/2 and PKB Requires Tyrosine Kinase Activity of Insulin Receptor and c-Src

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ABSTRACT

Hydrogen peroxide (H_2O_2) mimics many physiological responses of insulin, and increased H_2O_2 generation via the Nox-4 subunit of NAD(P)H oxidase was recently demonstrated to serve as a critical early step in the insulin signaling pathway. Exogenously added H_2O_2 has also been shown to activate several key components of the insulin signaling cascade. H_2O_2-induced signaling responses have been found to be associated with the activation of receptor and nonreceptor protein tyrosine kinases (PTK), including the insulin receptor (IR)-β subunit. Therefore, in the present studies on Chinese hamster ovary cells overexpressing wild-type IR-PTK (CHO-IR) or a PTK-inactive form of IR (CHO-1018), we investigated whether IR-PTK plays a role in H_2O_2-induced signaling events. Treatment of CHO-IR cells with H_2O_2 increased the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), protein kinase B (PKB), and glycogen synthase kinase-3β while enhancing tyrosine phosphorylation of the IR-β subunit and the p85 subunit of phosphatidylinositol 3-kinase (PI3K). Compared with CHO-IR cells, the stimulatory effect of H_2O_2 on ERK1/2 and PKB was partially reduced in CHO-1018 cells. However, pharmacological inhibition of Src family PTK by 4-amino-5-(4-chlorophenyl)-7-(*tert*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP-2) almost completely blocked H_2O_2-stimulated phosphorylation of the p85 subunit of PI3K, ERK1/2, and PKB. Moreover, H_2O_2, but not insulin, induced Tyr-418 phosphorylation of Src, which was also suppressed by PP-2. Taken together, these data suggest that both IR-PTK and Src family PTKs contribute to H_2O_2-induced signaling in CHO-IR cells albeit IR-PTK has a less dominant role in this process. *Antioxid. Redox Signal.* 7, 1014–1020.

INTRODUCTION

Reactive oxygen species have been suggested to serve as mediators of the action of several growth factors, cytokines, and insulin (5, 13, 20–22, 32). Insulin is the major hormone involved in glucose homeostasis, and its effect is initiated by binding to its receptor on cell membranes. The insulin receptor (IR) is a heterodimeric (α_2, β_2) protein, and insulin binding to the IR-α subunit results in conformational changes, leading to enhanced intrinsic protein tyrosine kinase (PTK) activity of the β subunit by multisite tyrosine phosphorylation. Once activated, IR-PTK can phosphorylate several cytosolic IR substrates such as IRSs and Shc, which serve as docking sites for Src homology 2 domain-containing signaling molecules (37), triggering the activation of two key signaling pathways. In one pathway, the association of IRS-1 with Grb-2-SOS complex results in activation of the Ras, Raf, MEK and extracellular signal-regulated kinase (ERK) pathway (36, 38). Activated ERK1/2 phosphorylates and activates a downstream ribosomal protein kinase, $p90^{rsk}$. Both ERK1/2 and $p90^{rsk}$ can be translocated to the nucleus where they phosphorylate transcription factors, and contribute the mitogenic and growth-promoting effects of insulin (36, 37). The second pathway that radiates from the IRS complex upon insulin stimulation involves phosphatidylinositol 3-kinase (PI3K) activation (36, 38). PI3K phosphorylates phosphatidylinositol

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(PI) lipids at position 3 of the inositol ring, and generates 3-phosphorylated forms of PI, such as phosphatidylinositol 3,4,5-trisphosphate (30), which are implicated in the activation of phospholipid-dependent kinase and related serine/threonine protein kinases. These activated kinases in turn are responsible for the phosphorylation and stimulation of several downstream signaling protein kinases, such as protein kinase B (PKB) (also known as Akt), glycogen synthase kinase 3 (GSK-3), $p70^{s6k}$ (11), and protein kinase C-ζ (PKCζ) (10). Activation of these protein serine/threonine kinases has been demonstrated to mediate the metabolic effects of insulin at the level of glucose transport, glucose transporter (GLUT-4) translocation, glycogen and protein synthesis (36).

Recent studies have demonstrated that insulin stimulation of cells generates a hydrogen peroxide (H_2O_2) burst that enhances the tyrosine phosphorylation of IR and IRS-1 (22). An important role for the NAD(P)H oxidase catalytic subunit homologue Nox-4 in enhancing H_2O_2 production in response to insulin has been suggested recently (23). In addition, exogenously added H_2O_2 has been shown to mimic many physiological effects of insulin, including glucose transport (15), glycogen synthesis (17), lipogenesis (24), lipolysis (19), and phosphoenolpyruvate carboxykinase gene expression (33). H_2O_2 has also been found to increase tyrosine phosphorylation of the IR-β subunit (15, 16) and activation of the ERK1/2 and PI3K/PKB signaling pathways in several cell types (3, 4, 6, 8, 18, 31, 34, 35). A potential role for epidermal growth factor (EGF) receptor transactivation (12, 28, 39) and the src family of PTK in H_2O_2-induced signaling has been postulated (1, 2, 12). However, despite the ability of H_2O_2 to enhance IR-PTK activity and IR-β subunit phosphorylation, the possible involvement of IR-PTK in H_2O_2-induced responses has not been characterized. Therefore, in the present studies, we investigated the possible requirement of IR-PTK in H_2O_2-induced phosphorylation of the ERK1/2 and PKB pathways.

MATERIALS AND METHODS

Materials

Insulin was obtained from Eli Lilly Co. (Indianapolis, IN, U.S.A.), H_2O_2 from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and IRS-1 and p85 antibodies from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Phospho-specific and total antibodies, against ERK1/2 and GSK-3β, antiphosphotyrosine antibody (PY99), and IR antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Phospho-specific and total PKB antibodies were procured from New England BioLabs (Beverly, MA, U.S.A.), and phospho-specific c-Src antibody from Biosource (Camarillo, CA, U.S.A.). Protein A Sepharose beads and enhanced chemiluminescence (ECL) detection kits were from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec, Canada).

Cell culture

Chinese hamster ovary (CHO) cells overexpressing either wild-type human IR (CHO-IR) or the PTK mutant form (CHO-1018), a gift from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA, U.S.A.), were maintained in Ham's F-12 medium containing 10% fetal bovine serum. They were grown to confluence in 100-mm plates and incubated in serum-free F-12 medium for 16 h prior to the experiment (26).

Cell lysis and immunoblotting

Cells subjected to various experimental treatments were washed twice with ice-cold phosphate-buffered saline and lysed in buffer A [25 m*M* Tris-HCl, pH 7.5, 25 m*M* NaCl, 1 m*M* sodium orthovanadate, 10 m*M* sodium fluoride, 10 m*M* sodium pyrophosphate, 20 m*M* okadaic acid, 0.5 m*M* ethylene-bis(oxyethylenenitrilo)tetraacetic acid, 1 m*M* phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1% Triton X-100]. The lysates were clarified by centrifugation to remove insoluble material. The clarified lysates, normalized to contain equal amounts of protein, were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, transferred to polyvinylidene difluoride (PVDF) membranes, and incubated with respective primary antibodies, followed by incubation with a horseradish peroxidase-conjugated second antibody. The antigen–antibody complex was visualized with an ECL detection kit. The immunoblots were quantified by densitometric scanning with NIH ImageJ software (27).

Immunoprecipitation

The clarified cell lysates, normalized to contain equal amounts of protein (500 μg), were immunoprecipitated overnight with 1 μg of PY99 antibody at 4°C, followed by incubation with protein A Sepharose for an additional 2 h. Immunoprecipitated phosphotyrosine proteins were collected by centrifugation, and washed twice with buffer A and once with phosphate-buffered saline. The phosphotyrosine protein immunoprecipitates were subjected to 7.5% SDS-PAGE under reducing conditions, transferred to PVDF membranes, and incubated with respective primary antibodies. Proteins were detected by a horseradish peroxidase-conjugated second antibody and visualized with an ECL detection kit (27).

PI3K assay

The clarified cell lysates were subjected to immunoprecipitation with 2 μg of p85α antibody for 2 h at 4°C, followed by incubation with protein A Sepharose for an additional 2 h. The immunoprecipitates were washed before PI3K assay, as described earlier (26). The phosphorylated lipid products were extracted and separated by ascending thin-layer chromatography (26). Radioactivity in the spots corresponding to PI 3-phosphate was quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS

Effect of H_2O_2 on ERK1/2, PKB, and GSK-3β phosphorylation in CHO-IR cells

We first evaluated whether H_2O_2 alters the activation of ERK1/2, PKB, and its downstream targets in CHO-IR cells. As increased phosphorylation in specific serine/threonine residues of these molecules is associated with their activa-

tion, their activity was measured by using phospho-specific antibodies. As shown in Fig. 1A, treatment of IR-overexpressing cells for 5 min with escalating concentrations of H_2O_2 induced ERK1/2, PKB, and GSK-3β phosphorylation. H_2O_2 increased ERK1/2 phosphorylation at concentrations as low as 10 μ*M* with maximal effect at 100 μ*M*, whereas higher concentrations (500 μ*M* to 1 μ*M*) were required to elicit robust PKB phosphorylation. Next, we assessed the time dependence of the H_2O_2 response. As seen in Fig. 1B, 1 m*M* H_2O_2 rapidly enhanced the phosphorylation of ERK1/2, PKB, and GSK-3β. The increase occurred within 5 min and then declined slowly to almost basal levels within 60 min of H_2O_2 treatment.

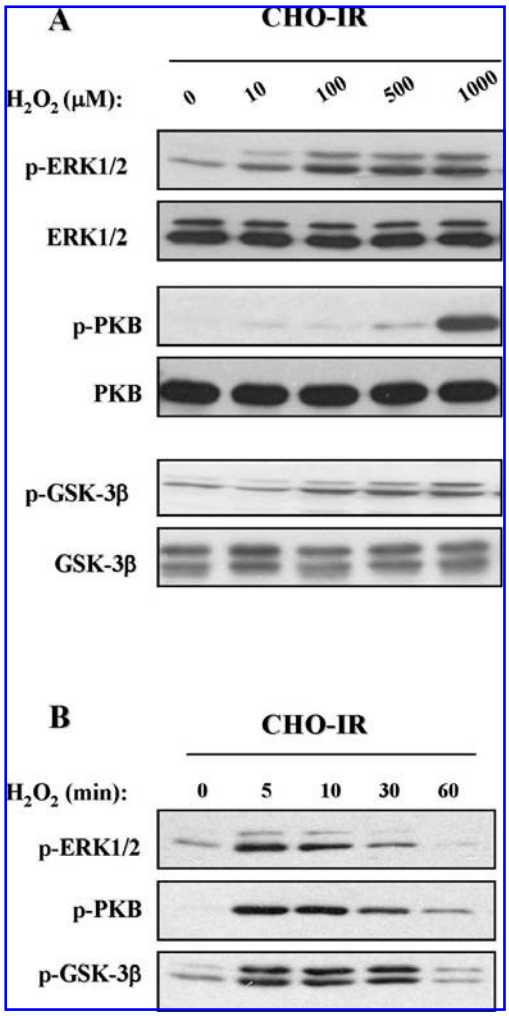

FIG. 1. H_2O_2-induced ERK1/2, PKB, and GSK-3β phosphorylation is time- and dose-dependent in CHO-IR cells. Confluent, serum-starved CHO-IR cells were incubated with different concentrations of H_2O_2 for 5 min (**A**) or with 1 m*M* H_2O_2 for the indicated time periods (**B**). The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Tyr-204)-ERK1/2 antibodies (upper immunoblot in A and B), phospho-specific (Ser-473)-PKB antibodies (middle immunoblot in A and B), and phospho-specific (Ser-9)-GSK-3β antibodies (lower immunoblot in A and B). The results are representative of three independent experiments.

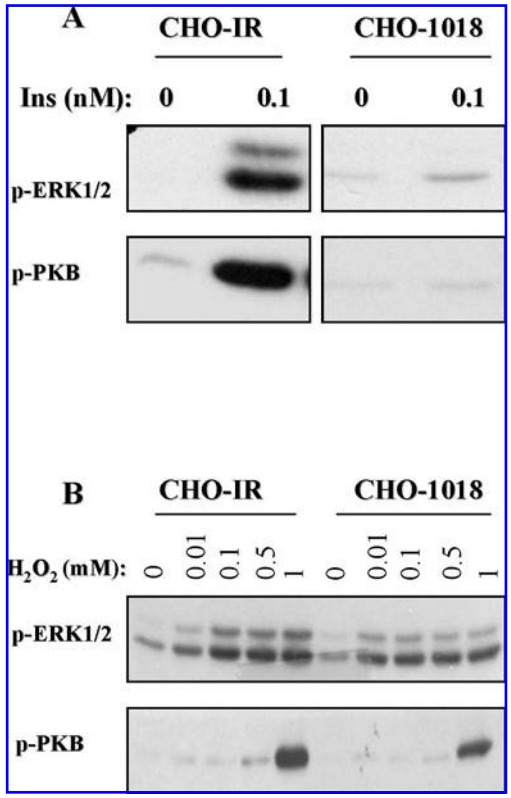

FIG. 2. H_2O_2-induced ERK1/2 and PKB phosphorylation is partially dependent on IR-PTK activity in CHO cells. Confluent, serum-starved CHO-IR and CHO-1018 cells were incubated in the absence or presence of 0.1 n*M* insulin (Ins) (**A**) or with different concentrations of H_2O_2 (**B**) for 5 min. The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Tyr-204)-ERK1/2 antibodies (upper immunoblot in A and B) and phospho-specific (Ser-473)-PKB antibodies (lower immunoblot in A and B). The results are representative of three independent experiments.

Effect of H_2O_2 on ERK1/2 and PKB phosphorylation in IR-PTK-deficient cells

To examine the role of IR-PTK in H_2O_2-induced effects on ERK1/2 and PKB phosphorylation, we studied CHO-1018 cells that overexpress an inactive form of IR-PTK. The inactive form of IR was generated by the mutation of lysine 1018 to alanine in the ATP-binding domain of the IR-β subunit. This mutation results in the loss of ATP-binding activity and, thus, PTK function (7). As illustrated in Fig. 2, both insulin and H_2O_2 enhanced the phosphorylation of ERK1/2 and PKB in CHO-IR cells overexpressing a normal IR. However, in CHO-1018 cells, the insulin-induced phosphorylation of both ERK1/2 and PKB was almost completely attenuated, whereas the H_2O_2-evoked increase was partially blocked in this cell type. These data suggested that, compared with insulin, H_2O_2-induced phosphorylation of ERK1/2 and PKB is exerted in part through IR-PTK activity.

Effect of H_2O_2 on IR, IRS-1, and p85 tyrosine phosphorylation

As the insulin effects on ERK1/2 and PKB are mediated through the enhanced tyrosine phosphorylation of IR and IRS-1, we next explored if, similarly to insulin, H_2O_2 increases the tyrosine phosphorylation of IR and IRS-1. As shown in Fig. 3, both insulin and H_2O_2 augmented tyrosine phosphorylation of the IR-β subunit, but tyrosine phosphorylation of the IR-β subunit induced by H_2O_2 was significantly lower than that observed with insulin. Furthermore, whereas insulin enhanced the tyrosine phosphorylation of IRS-1, a similar effect of H_2O_2 on IRS-1 tyrosine phosphorylation was not detected in CHO-IR cells (Fig. 3). In contrast, both insulin and H_2O_2 treatment resulted in enhanced tyrosine phosphorylation of the p85 subunit of PI3K.

Requirement of c-Src in H_2O_2-induced ERK1/2 and PKB phosphorylation

The results with CHO-1018 cells indicated that the H_2O_2 effect on ERK1/2 and PKB phosphorylation required additional signals besides IR-PTK. As potential involvement of the Src family of PTKs in H_2O_2-induced signaling has been proposed in other cell types, we investigated if, in CHO cells also, Src-PTK was responsible for triggering the phosphorylation of various signaling components. We utilized PP-2 [4-amino-5-(4-chlorophenyl)-7-(*tert*-butyl)pyrazolo[3,4*d*] pyrimidine], a selective inhibitor of Src-PTK, and PP-3 (4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine), an inactive analogue of PP-2, to examine the contribution of Src in H_2O_2-induced increased phosphorylation events. As depicted in Fig. 4, treatment of cells with PP-2 almost completely suppressed H_2O_2-stimulated phosphorylation of both ERK1/2 and PKB. As

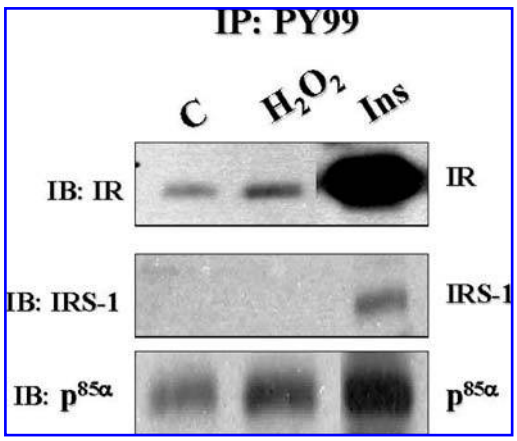

FIG. 3. H_2O_2-induced tyrosine phosphorylation of IR and p85, but not IRS-1, in CHO-IR cells. Confluent, serum-starved CHO-IR cells were incubated with 100 n*M* insulin (Ins) or with 1 m*M* H_2O_2 for 5 min. The cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with an antiphosphotyrosine (PY99) antibody. The immunoprecipitates were immunoblotted with the indicated antibodies. A representative immunoblot (IB) from three independent experiments is shown.

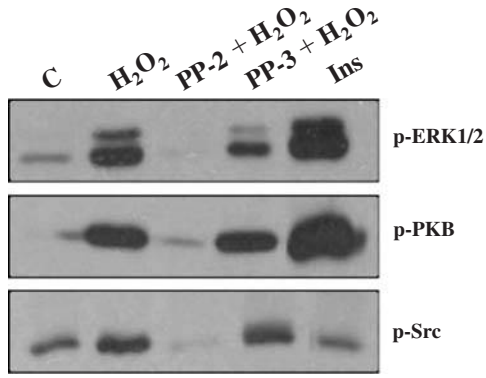

FIG. 4. H_2O_2-induced ERK1/2 and PKB phosphorylation is totally dependent on Src-PTK activity in CHO-IR cells. Confluent, serum-starved CHO-IR cells were pretreated with or without 10 μ*M* PP-2 or its inactive analogue PP-3 for 30 min, followed by incubation with 1 m*M* H_2O_2 or 100 n*M* insulin (Ins) for 5 min. The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Tyr-204)-ERK1/2 antibodies (upper immunoblot), phospho-specific (Ser-473)-PKB antibodies (middle immunoblot), and phospho-specific (Tyr-418)-Src antibodies (lower immunoblot). The results are representative of three independent experiments.

PP-2 is a potent and specific inhibitor of c-Src activity, and its ability to suppress H_2O_2-induced responses suggested a role of Src in this process, we directly assessed if H_2O_2 would enhance Src activity in CHO-IR cells. This was achieved by evaluating the increase in Tyr-418 phosphorylation in the activation loop of c-Src. As shown in Fig. 4, H_2O_2 treatment enhanced the Tyr-418 phosphorylation of Src, which was almost completely blocked in PP-2-pretreated cells.

Effect of PP-2 on IR and p85 tyrosine phosphorylation and of wortmannin on ERK1/2 and PKB phosphorylation induced by H_2O_2

To determine if c-Src is an upstream mediator of IR and p85 tyrosine phosphorylation, we evaluated the effect of PP-2 on H_2O_2-induced phosphorylation of these signaling components. As illustrated in Fig. 5A, PP-2 treatment failed to block tyrosine phosphorylation of the IR-β subunit, whereas it completely attenuated the tyrosine phosphorylation of the p85 regulatory subunit of PI3K. Next, we examined if p85 phosphorylation by H_2O_2 was associated with an increase in the PI3K activity of CHO-IR cells. As seen in Fig. 5B, H_2O_2 treatment enhanced PI3K activity, which was sensitive to inhibition by wortmannin, a specific PI3K inhibitor. Furthermore, both ERK1/2 and PKB phosphorylation induced by H_2O_2 were almost completely attenuated by wortmannin pretreatment of cells (Fig. 5C).

DISCUSSION

In this study, we have shown that exogenously added H_2O_2 enhanced the phosphorylation of ERK1/2 and PKB signaling pathways in CHO-IR cells. The increased phosphorylation of

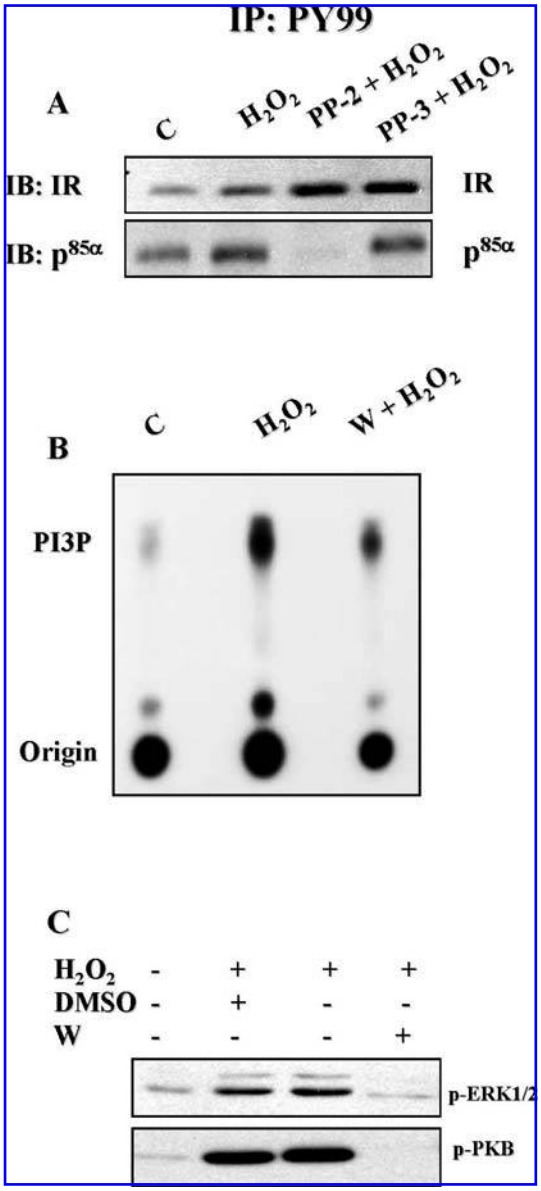

FIG. 5. H_2O_2-induced p85 phosphorylation and ERK1/2 and PKB phosphorylation are dependent on Src-PTK and PI3K, respectively. Confluent, serum-starved CHO-IR cells were pretreated with 10 μ*M* PP-2 or its inactive analogue PP-3 (**A**) or 100 n*M* wortmannin (W) (**B** and **C**) for 30 min, followed by incubation with 1 m*M* H_2O_2 for 5 min. In A, the cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with antiphosphotyrosine (PY99) antibody. The immunoprecipitates were immunoblotted (IB) with IR antibodies (upper immunoblot) and p85 antibodies (lower immunoblot). In B, the cells were lysed, and the cell lysates were subjected to immunoprecipitation using p85α antibodies. PI3K activity was measured in p85α immunoprecipitates with PI as substrate and [γ-^{32}P]ATP as phosphoryl group donor. The position of PI 3-phosphate (PI3P) is indicated. In C, the cells were lysed and the lysates were subjected to immunoblotting using phospho-specific (Tyr-204)-ERK1/2 antibodies (upper immunoblot) and phospho-specific (Ser-473)-PKB antibodies (lower immunoblot). DMSO, dimethyl sulfoxide. The results are representative of three independent experiments.

ERK1/2 and PKB was associated with heightened tyrosine phosphorylation of the IR-β subunit and p85 subunit of PI3K. We have also provided evidence that PTK activity of the IR-β subunit is partially responsible for H_2O_2-induced activation of the ERK1/2 and PKB pathways. This contention is based on the results with the CHO cells that overexpress a PTK-inactive form of IR. Although earlier studies have demonstrated a stimulatory effect of H_2O_2 on tyrosine phosphorylation of the IR-β subunit in adipocytes, no attempts have been made to determine the contribution of IR-PTK in H_2O_2-induced signaling events. Thus, to the best of our knowledge, this work provides the first evidence in support of the involvement of IR-PTK in H_2O_2-induced phosphorylation of ERK1/2 and PKB.

H_2O_2 has recently emerged as an important redox molecule for the action of several growth factors, cytokines, and insulin (5, 13, 20–22, 32). Some of the effects of H_2O_2 on signaling events have been shown to be mediated by transactivation of EGF receptor PTK and the Src family of PTKs in several cell types (1, 2, 12, 28, 39). In CHO-IR cells, H_2O_2 also enhanced Src activity, as judged by the increased phosphorylation of Tyr-418 in the activation loop of Src. The participation of Src in H_2O_2-induced signaling in CHO cells was evaluated by using PP-2, a highly specific inhibitor of Src PTK activity. PP-2 almost completely attenuated the H_2O_2-induced increase of phosphorylation of ERK1/2, PKB, and the p85 subunit of PI3K. This is in contrast to the partial inhibition elicited in IRPTK-deficient cells where the H_2O_2-induced effect was blocked by only 20–30% (Fig. 2). An important role of Src PTK in insulin-induced activation of PKCδ was demonstrated recently in primary cultures of skeletal myotubes (29). In these studies, insulin stimulated Src PTK activity, which was blocked by PP-2 (29). Moreover, PP-2 also inhibited insulin-induced IR-β tyrosine phosphorylation, as well as glucose uptake in these myotubes. In the case of CHO-IR cells, however, neither insulin- nor H_2O_2-induced IR-β subunit phosphorylation was blocked by PP-2. Furthermore, PP-2 was ineffective in inhibiting the increased phosphorylation of ERK1/2 and PKB induced by insulin (data not shown), whereas it completely blocked the H_2O_2-evoked effect in these cells (Fig. 4), which suggested that Src-PTK-dependent pathways in CHO-IR cells mediate the H_2O_2 response and not that of insulin. Furthermore, in contrast to insulin, H_2O_2 treatment did not enhance IRS-1 tyrosine phosphorylation, but increased tyrosine phosphorylation of the p85 subunit of PI3K, which was sensitive to inhibition by PP-2. Src family PTK-catalyzed Tyr-688 phosphorylation of p85 was recently shown to activate PI3K in COS cells (9). The fact that H_2O_2 induced PI3K activation in these cells, and wortmannin, a specific inhibitor of PI3K, blocked H_2O_2-evoked activation of ERK1/2 and PKB in these cells, suggests that PI3K may be an upstream intermediate in H_2O_2-induced signaling events.

Recent studies have shown that insulin-induced generation of H_2O_2 via NAD(P)H oxidase activation serves as a trigger to initiate insulin signaling (23). In these experiments, ablation of Nox-4, one of the subunits of NAD(P)H oxidase complex, resulted in diminished H_2O_2 production, associated with decreased tyrosine phosphorylation of IR and IRS-1 (23). The data indicated that endogenously generated H_2O_2 in response to insulin is able to enhance IRS-1 phosphorylation in 3T3-L1 adipocytes (23), whereas the results with CHO-IR cells dem-

onstrated that exogenous H_2O_2 fails to modify IRS-1 phosphorylation (Fig. 3). Thus, it appears that the intracellular upstream targets of endogenously generated and exogenously added H_2O_2 may be different.

The precise mechanism by which H_2O_2 induces tyrosine phosphorylation of substrate proteins remains obscure; however, its ability to inhibit the activities of many protein tyrosine phosphatases (PTPases), such as PTP1B (18), and SHP-2 (25), has been suggested as a potential mechanism. PTPase inhibition by H_2O_2 is accomplished by oxidation of catalytically essential cysteine residue in the active site of PTPase (14); this inhibition shifts the equilibrium of the phosphorylation–dephosphorylation cycle, resulting in increased tyrosyl phosphorylation of substrate proteins, such as Src, the IR-β subunit, and the p85 subunit of PI3K. These tyrosyl-phosphorylated proteins promote the assembly of signaling molecules responsible for activating various components of the mitogen-activated protein kinase and PI3K signaling pathways.

In summary, our studies have demonstrated that H_2O_2-induced activation of ERK1/2 and PKB is associated with enhanced tyrosine phosphorylation of the IR-β subunit, the p85 subunit of PI3K, and c-Src in CHO-IR cells. We have also provided evidence of the involvement of both IR-PTK- and Src-PTK-dependent upstream mechanisms as mediators of the H_2O_2 response.

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ABBREVIATIONS

CHO, Chinese hamster ovary; CHO-IR, Chinese hamster ovary cells overexpressing insulin receptor; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinases; GSK, glycogen synthase kinase; H_2O_2, hydrogen peroxide; IR, insulin receptor; IRS, insulin receptor substrate; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PP-2, 4-amino-5-(4-chlorophenyl)-7-(*tert*-butyl)pyrazolo[3,4-*d*]pyrimidine; PP-3, 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine; PTK, protein tyrosine kinase; PTPases, protein tyrosine phosphatases; PVDF, polyvinylidene difluoride; PY99, antiphosphotyrosine antibody; SDS, sodium dodecyl sulfate.

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