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# Tyrphostin AG1478 Accelerates Hydrogen Peroxide-Induced Apoptosis in A431 Cells

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**Oxidative stress is a potent inducer of apoptosis and activates protein tyrosine kinases and cytokine receptors, such as the epidermal growth factor receptor (EGFR). Previous studies suggest that cytokine receptors are potential effectors for anti-apoptotic signals, but it has not previously been determined whether cytokine receptors regulate down-stream protein kinases. To investigate the role of EGFR on oxidative stress-induced apoptosis and its down-stream protein kinases, we blocked EGFR activation with Tyrphostin AG1478, a highly selective EGFR inhibitor. We determined that Tyrphostin AG1478 accelerated hydrogen peroxide-induced apoptosis in A431 cells, with activation of caspases 3 and 9, and decreased mitochondrial membrane potential. Hydrogen peroxide induced-activation of EGFR, Akt/PKB, MAPK, and Bad (both Ser-112 and Ser-136 residues) were inhibited by Tyrphostin AG1478. These results suggest that early upstream signaling events, such as EGFR activation, exert anti-apoptotic effects by regulating MAPK, Akt/PKB, and phosphorylation of Bad.**

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**Key Words:** *EGFR, $H_2O_2$, Tyrphostin AG1478, apoptosis*

## Introduction

Oxidative stresses are known to induce apoptosis. On the other hand, it is also recognized that oxidative stresses activate a number of protein kinases, including PI3 - kinase, Akt / PKB[1-3], MAPK[4-6], p38 MAPK[7-8], and JNK[9, 10] and cytokine receptors, such as EGFR[11-14] Results of recent studies indicate that activation of MAPK and PI3 - kinase - Akt / PKB participate in cell death induced by oxidative stress[3, 4]. Under certain circumstances, apoptosis may be controlled by at least two distinctive anti-apoptotic signals, the PI3 - kinase - Akt / PKB, and MAPK pathways. It is also known that non - small cell lung cancer (NSCLC), that overexpress EGFR are resistant to anti-cancer drugs[15-17]. The results demonstrate that blocking EGFR activation by Tyrphostin AG1478, augmented hydrogen peroxide - induced apoptosis, which was accompanied by activation of both caspase 9, 3, and decreased mitochondrial membrane potential and marked reduced activation of MAPK, Akt / PKB, and a downstream target, Bad (both Ser-112 and Ser-136). These results suggest that activation of cytokine receptors, such as EGFR exert potential anti - apoptotic effects by regulating MAPK and via two distinctive pathways that phosphorylate both Ser - 112 and 136 residues of Bad.

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## Materials and Methods

*Reagents.*

Hydrogen peroxide ($H_2O_2$) was purchased from Wako (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from GIBCO (Grand Island, NY). Propidium iodide (PI) was purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal anti-phospho-Erk antibody, polyclonal anti-Erk 1/2 antibody, polyclonal anti-phospho-Akt/PKB antibody, polyclonal anti-Akt/PKB antibody and polyclonal anti-phospho-Bad (Ser-136, Ser-112) antibody were purchased from New England Biolabs (Beverly, MA). Monoclonal anti-caspase 9 antibody was obtained from MBL (Nagoya, Japan). Polyclonal anti-caspase 3 antibody was obtained from PharMingen. (San Diego, CA). Monoclonal anti-EGFR antibody and anti-Bad antibody were purchased from Transduction Lab (Lexington, KY). Monoclonal anti-phospho-EGFR antibody was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

*Cell culture.*

A431 cells were obtained from ATCC and cultured in plastic dishes with DMEM supplemented with 10 % FBS at 37℃ in 5% $CO_2$ / 95% air gas mixture. A431 / wt PDGFR - beta cells[18] were cultured as previously described.

*Apoptosis Assays.*

Apoptosis was analyzed in two ways :1) staining cells with the potential sensitive fluorescent dye $DiOC_6(3)$ (3,3'-dihexyloxacarbocyanine iodide) from Lambda Co. (Graz, Austria) to evaluate mitochondrial $\Delta\Psi m$ as previously reported[19, 20]. Briefly, cells were stained for 15 min at 37℃ with 40 nM of $DiOC_6(3)$. After washing once with PBS, the decrement of mitochondrial membrane potential was measured by flow cytometry. 2) For hypodiploid cell assessment, cells ($5X10^5$) were washed twice in PBS and fixed overnight in ethanol (70% in water, at 4℃). Cells were then resuspended in 0.5 ml of PBS containing 50 $\mu$g/ml propidium iodide (PI) (Sigma) and incubated for 30 min at 4℃ The DNA content of $10^4$ cells was monitored by flow cytometry[21].

*Western blot analysis.*

A431 cells (9 x $10^5$) were grown to subconfluence on 6 well culture dishes and starved by serum - free medium containing 1% BSA for 24 h. The cells were treated with dimethyl sulfoxide (0.15% : vol/vol) or indicated concentrations of Tyrphostin AG1478 (0.05, 0.15, 0.5, 1.5, 5, 15 $\mu$M ; 0.15% : vol/vol) prior to stimulation with hydrogen peroxide (0.5 mM) for the indicated times (0, 5, 10, 15, 30, 60, 90, 120, 180 min). A431 cells were washed with cold PBS and lysed by the addition of lysis buffer (1% NP-40, 50 mM Tris, [ pH 7.5 ], 150 mM NaCl, 1 mM EDTA, 1.0 mM PMSF, 5 $\mu$g/ml aprotinin, 5 $\mu$g/ml leupeptin, 5 $\mu$M phenylmethyl sulfoxide) for 20 min at 4℃, and insoluble materials were removed by centrifugation at 13,000 x g for 20 min at 4℃. The supernatant was collected and the protein concentration was determined by the Bio-Rad protein assay kit. An identical amount of protein (20 $\mu$g) for each lysate was subjected to 6~15% SDS - PAGE. For investigation of Bad phosphorylation, proteins were separated on low bis-acrylamide gels. Proteins were transferred to a PVDF membrane, and the filters were blocked for 1.5 h using 5% bovine serum albumin in TBS (50 mM Tris, 0.15 M NaCl, pH 7.5) containing 0.1 % Tween 20, washed with TBS and incubated at room temperature for 2 h in an indicated dilution of antibody. Filters were later washed with TBS and incubated with 1 : 1000 dilution of donkey anti-mouse IgG antibody or 1 : 5000 goat-anti-rabbit IgG antibody coupled with horseradish peroxidase. The enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, MA) was used for detection. Filters were subsequently exposed to film for 15 s and the latter was processed.

*Immunoprecipitation*

Cell lysates (200 $\mu$g/protein) were immunoprecipitated with anti-Bad antibody (4 $\mu$g) for 6 h and then 20 $\mu$l of protein G (50% ; vol/vol) was added and shaken for 1 hr. After washing 3 times with lysis buffer, 20 $\mu$l of 1 x reducing buffer was added to protein G and boiled and samples were collected.Statistics. All values were expressed as mean ± S.E.M. of n experiments. Data were analyzed using the paired t-test. A p value $< 0.05$ denoted statistical significance.

## RESULTS

*Tyrphostin AG1478 accelerates hydrogen peroxide induced apoptosis accompanied by caspase 9 and 3 activation and decreased mitochondrial membrane potential in A431 cells.*

Previous study demonstrated the potency of EGFR as anti-apoptotic effect for oxidative stresses by phosphorylation of downstream protein kinases, such as Akt / PKB[3] or MAPK[4] and prevent apoptosis. To determine whether blockade of EGFR activation leads to acceleration of hydrogen peroxide-induced apoptosis, we therefore investigated whether blockade of EGFR activation leads to acceleration of hydrogen peroxide-induced apoptosis. Using flow cytometry with propidium iodide staining, we determined the optimal concentration of hydrogen peroxide and exposure time for induction of apoptosis. The increased induction of apoptosis was observed in a time and dose dependent manner (data not shown). To determine the effect of Tyrphostin AG1478 on apoptosis induced by hydrogen peroxide, we investigated the apoptosis ratio in 0.5 mM of hydrogen peroxide insults for 30 h with or without the inhibitor. As shown in Figure 1 A, at concentration of Tyrphostin AG1478 $\geq$ 5 $\mu$M, significantly increased apoptosis was observed compared with control (incubated with DMSO and $H_2O_2$ : 0.5 mM).

Recent reports suggest that hydrogen peroxide induced - apoptosis is accompanied by caspase 3 activation secondary to mitochondrial injury and release of cytochrome C to the cytosol[22]. We utilized western

blotting to examine activation of caspase 9 and 3 in response to hydrogen peroxide. As shown in Figure 1B, hydrogen peroxide - induced activation of caspase 9 and 3 was increased with Tyrphostin AG1478 in a dose dependent manner. Recently several reports have suggested that mitochondrial membrane potential is correlated with mitochondrial injury[19, 20]. Using flow cytometry with $DiOC_6(3)$, we investigated the mitochondrial membrane potential in response to hydrogen peroxide in the absence or presence of Tyrphostin AG1478. As shown in Figure 1C, Tyrphostin AG1478 significantly decreased the mitochondrial membrane potentials in a dose dependent manner.

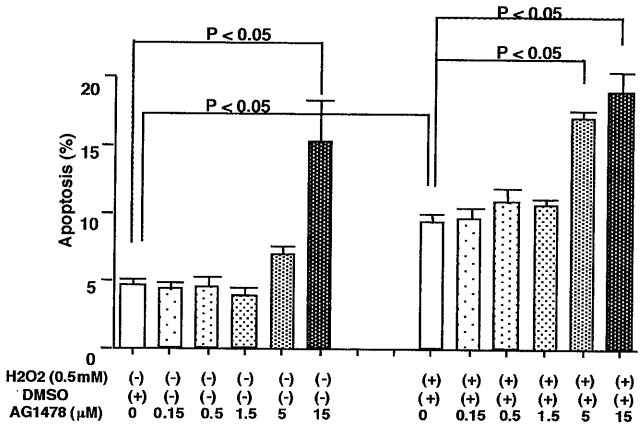


**Figure 1A.** Effect of Tyrphostin AG1478 on hydrogen peroxide - induced apoptosis. Cells were treated with DMSO or with Tyrphostin AG1478 at the indicated concentrations for 30 min prior to stimulating with hydrogen peroxide (0.5 mM) for 30 h under serum - free conditions (1% BSA). Cells were collected and analyzed by flow cytometry with propidium iodide staining and the apoptosis ratio was investigated. All experiments were repeated at least three times with similar results.

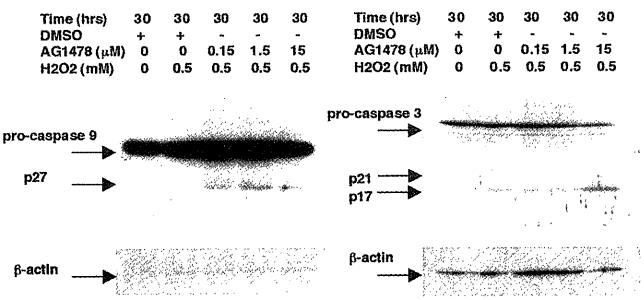


**Figure 1B.** Effect of Tyrphostin AG1478 on the activation of caspase 9 and 3 by hydrogen peroxide. Quiescent cells were treated with DMSO or Tyrphostin AG1478 as indicated for 30 min prior to inducing apoptosis with hydrogen peroxide (0.5 mM) for 30 h under serum - free condition. Cell lysates (20 $\mu$g of protein) were used in Western blots with anti - caspase 9, and 3 antibodies and anti - $\beta$ actin antibody. All experiments were repeated at least three times with similar results.

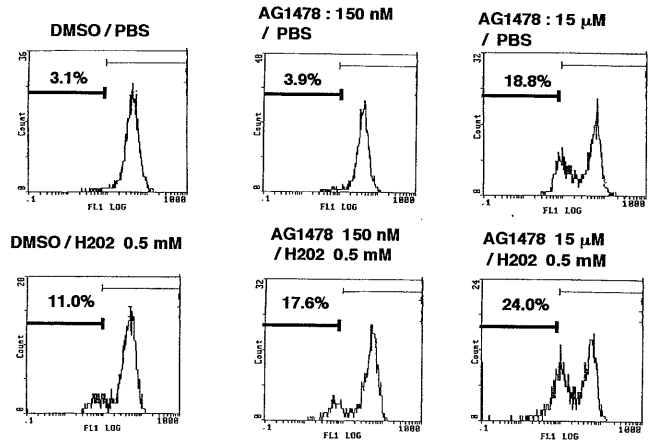


**Figure 1C.** Effect of Tyrphostin AG1478 on trans - membrane potential by hydrogen peroxide. Cells were treated with DMSO or Tyrphostin AG1478 at the indicated concentrations for 30 min prior to stimulating with hydrogen peroxide (0.5 mM) for 30 h under serum - free conditions (1% BSA). Cells were collected and analyzed by flow cytometry with $DiOC_6(3)$ staining, and mitochondrial membrane potential was investigated. Numbers indicate the percentage of gated cells displaying decreased mitochondrial membrane potential. All experiments were repeated at least three times with similar results.

*Hydrogen peroxide activates EGFR, Akt / PKB, MAPK, and Bad in A431 cells.*

It has been reported that cytokine receptors, such as EGFR[11-14] and PDGFR[23] are activated by oxidative stress, as well as protein kinases activation, such as MAPK, PI3 - kinase, and Akt / PKB. We investigated the activation and phosphorylation of EGFR, PI3 - kinase, Akt / PKB, MAPK, and Bad (Ser - 112, Ser - 136) in response to hydrogen peroxide in A431 cells. As reported previously, activation of EGFR was observed in response to hydrogen peroxide and was sustained for more than 180 min (Figure 2A). As shown in Figure 2 B, hydrogen peroxide addition activated Akt / PKB and activation of Akt / PKB reached a plateau level by 60 min and was sustained for more than 180 min. Next, we investigated the time course of MAPK activation. As shown in Figure 2 C, hydrogen peroxide led to MAPK activation, and this activation was also sustained for more than 180 min. Finally, we investigated the time course of phosphorylation of Bad, Both the Ser - 112 and Ser - 136 residue of Bad was phosphorylated and this phosphorylation was sustained for more than 180 min (Figure 2D).

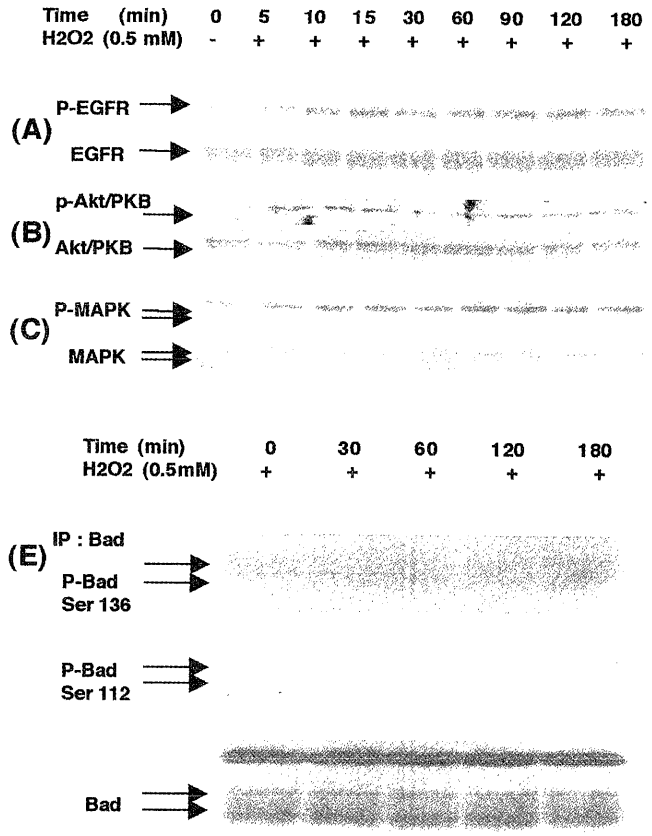


**Figure 2.** Effect of hydrogen peroxide on the activation of EGFR, PI3 - kinase, Akt / PKB, MAPK, and phosphorylation of Bad (ser-136, ser-112) Quiescent cells were stimulated with hydrogen peroxide (0.5 mM) for various times as indicated. Cell lysates (20 $\mu$g of protein) were prepared and analyzed by immunoblotting. (A) phosphorylated EGFR and EGFR; (B) phospho - Akt / PKB and Akt / PKB; (C) phospho - MAPK and MAPK; (D) Lysates (200 $\mu$g of protein) were prepared and immunoprecipitated with anti - Bad monoclonal antibody, and the anti-Bad immunoprecipitates were analyzed by Western blots with anti-phosphorylated Bad (Ser-136), anti-phosphorylated Bad (Ser-112) and anti - Bad antibody. All experiments were repeated at least three times with similar results.

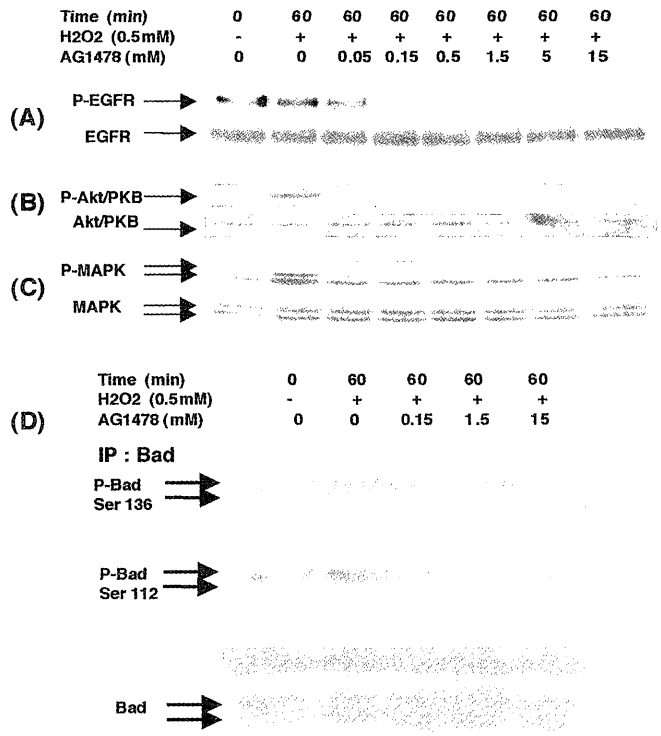


**Figure 3.** Effect of Tyrphostin AG1478 on hydrogen peroxide - induced activation of EGFR, PI3 - kinase, Akt / PKB, MAPK, and phosphorylation of Bad (ser-136, ser-112). Quiescent cells were treated with DMSO or Tyrphostin AG1478 at various concentrations as indicated for 30 min prior to stimulation with hydrogen peroxide (0.5 mM) for 60 min. Cell lysates (20 $\mu$g) were prepared and analyzed by immunoblotting. (A) phosphorylated EGFR, and EGFR; (B) phospho - Akt / PKB, and Akt / PKB; (C) phospho - MAPK, and MAPK; (D) Lysates (200 $\mu$g of protein) were prepared and immunoprecipitated with anti - Bad monoclonal antibody, and the anti - Bad immunoprecipitates were also analyzed by Western blots with anti-phosphorylated Bad (Ser-136), anti - phosphorylated Bad (Ser-112) and anti - Bad antibody. All experiments were repeated at least three times with similar results.

*Tyrphostin AG1478 inhibited hydrogen peroxide induced phosphorylation of EGFR, Akt / PKB, MAPK, and Bad.*

Using western blot analysis, we investigated the effect of Tyrphostin AG1478 on hydrogen peroxide-induced EGFR stimulation. Tyrphostin AG1478 inhibited EGFR activation by hydrogen peroxide in a dose dependent manner (Figure 3A). At the time of 60 min, a similar inhibitory effect of Tyrphostin AG1478 on the activation of Akt / PKB (Figure 3B) and MAPK (Figure 3C). Finally we investigated the effect of Tyrphostin AG1478 on the phosphorylation of Bad at the time of 60 min. Phosphorylation of both serine residues (Ser-136, and Ser-112) of Bad in response to hydrogen peroxide was significantly prevented (Figure 3D).

*Confirmation of a specificity of Tyrphostin AG1478 for EGFR.*

Tyrphostin AG1478 is a member of the class of quinazoline inhibitors of the EGFR tyrosine kinases. It blocks EGFR phosphorylation and Src kinase activity *in vivo* but does not inhibit the PDGFR[24-27]. To determine whether the observed inhibitory effects of Tyrphostin AG1478 on the activation of downstream protein kinases were mediated through inhibition of EGFR, we investigated the effect of Tyrphostin AG1478 on the activation of MAPK and Akt / PKB in response to PDGF - BB. As reported previously, A431 cells lack endogenous PDGFR[18]. Using the A431 / wt PDGFR beta cells in which wild type PDGFR beta

was introduced[18], we stimulated A431 / wt PDGFR beta cells with PDGF BB with or without indicated concentrations of Tyrphostin AG1478 (150 nM, 1.5 $\mu$M, 15 $\mu$M ), for 15 min. As shown in Figure 4A, activation of MAPK by PDGF - BB was slightly prevented at 15 _M of Tyrphostin AG 1478, but even at 15 _M of Tyrphostin AG1478, Akt / PKB activation was unaffected (Figure 4B).

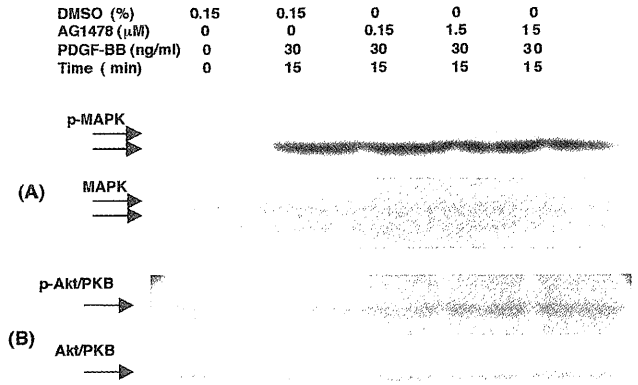


**Figure 4.** Effect of Tyrphostin AG1478 on PDGF - BB stimulated MAPK activation in A431/wt PDGF beta cells. Quiescent A431/wt PDGF beta cells were treated for 30 min with DMSO or Tyrphostin AG1478 at various concentration as indicated prior to stimulation with PDGF BB (30 ng/ml) for 15 min. Cell lysates (20 $\mu$g of protein) were prepared and analyzed by immunoblotting. (A) phosphorylated MAPK, MAPK. (B) phosphorylated Akt/PKB, Akt/PKB. All experiments were repeated at least three times with similar results. ABBREVIATION EGF : epidermal growth factor, EGFR : epidermal growth factor receptor, PDGF: platelet-derived growth factor, PDGFR : platelet-derived growth factor receptor, MAPK : mitogen-activated protein kinase, JNK : C-jun N terminal kinase, P38MAPK : P38 mitogen-Activated protein kinase, PI3K : phosphoinositide 3-kinase, Akt/PKB : Akt/protein kinase B, PKC : protein kinase C, PKB : protein kinase B, MEK : mitogen-activated protein kinase kinase

## DISCUSSION

Oxidative stress elicits rapid, highly regulated adaptive responses involving cordinate control of multiple signal transduction pathways. Previous studies suggested that early signal transduction events after oxidative stress appear to occur at or near the cell membrane[28]. However, there are few studies that have directly addressed the consequences of the activation of cytokine receptors and their cordination of downstream kinases in response to oxidative insults. The aim of the present study was to examine possible anti - apoptotic effects of cytokine receptors. We focused upon EGFR activation by hydrogen peroxide and used A431 cells, which have abundant EGFRs.

From our results, the following conclusions were obtained : 1) In A431 cells, Tyrphostin AG1478 accelerated hydrogen peroxide - induced apoptosis, which was accompanied by activation of caspases 3 and 9 and decreased mitochondrial membrane potential ; 2) hydrogen peroxide induced sustained activation of EGFR, Akt / PKB, MAPK, and Bad (both Ser-112 and Ser-136 residue), and activation of EGFR, Akt / PKB, MAPK, and Bad with hydrogen peroxide were inhibited by Tyrphostin AG1478 in a dose dependent manner, 3) The effect of Tyrphostin AG1478 on the EGFR activation was specific, since there were no effects upon PDGFR-mediated activation of MAPK, Akt / PKB.

Yang E. et al demonstrated that Bad ( Ser - 136 ) is one of the targets of Akt / PKB activation; in addition, Bad (Ser - 112) is a target of P90 RSK which is thought to be located downstream of PKC[29-34] and MAPK[35-36]. Therefore the results of the present studies suggest that one mechanism of accelerated hydrogen peroxide-induced apoptosis may be the result of accelerating caspase activation by mitochondrial injury due to prevention of Bad phosphorylation.

It is known that administration of a MEK inhibitor, PD98059 or a PI3 - kinase inhibitor, Wortmannin will accelerate oxidative stress- induced apoptosis[3, 4]. These previous studies suggest that upstream kinases, such as MEK or PI3 - kinase partially regulates anti-apoptotic downstream protein kinases, such as MAPK or Akt / PKB in response to oxidative stress. On the other hands, phosphorylation of these protein kinases and cytokine receptors has been suggested to be due to inactivation of phosphatases by oxidative stress[37]. It was unclear whether EGFR cordinate these downstream kinses in the previous studies with Tyrphostinn AG1478. However, it is still unclear whether the cytokine receptors systemically regulate anti - apoptotic protein kinases, such as MAPK or PI3 - kinase. In regard to the signaling system, there are two different scenarios concerning the activation of protein kinases in response to oxidative stresses. The first scenario is that once cytokine receptors are activated in response to oxidative stress and regulate their downstream protein kinases systemically, which protect the cells from apoptosis. The second scenario is that in spite of partial regulation of protein kinases by upstream kinases, each protein kinase and each receptor is activated at random due to inactivation of specific phosphatases. As expected, Tyrphostin AG1478 inhibited the activation of MAPK, PI3 - kinase, and Akt / PKB, as well as EGFR activation. Tyrphostin AG1478 also inhibited the phosphorylation of both serine residues of Bad in response to hydrogen peroxide. Therefore, our results

support the first scenario and suggest that EGFR regulates downstream protein kinases systemically and prevents apoptosis by phosphorylation of Bad.

Tyrphostin AG1478 has been reported to be highly selective as an EGFR blocker[24-27]. In the present study, we examined whether Tyrphostin AG1478 would inhibit the activation of protein kinases in response to a different, structurally dissimilar receptor tyrosine kinase. We utilized A431 / wt PDGFR beta cells, in which wild type PDGFR beta was introduced[18]. Activation of Akt / PKB by PDGF - BB was not affected by Tyrphostin AG1478, and activation of MAPK, by PDGF - BB was only slightly affected by Tyrphostin AG1478. From these results, we conclude that Tyrphostin AG1478 does not have any non - specific inhibitory effect for Akt / PKB activation but cannot rule out that it may have some minor, non - specific inhibitory effect for MAPK activation.

Therefore, in conclusion, we demonstrate that EGFR is involved in, and regulates cell survival signaling pathways, such as MAPK and Akt / PKB and its downstream target, Bad in response to oxidative insults. These results suggest potential anti - apoptotic effects of cytokine receptors by regulating downstream kinases in response to hydrogen peroxide.

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