Protein Kinase B Activation by Reactive Oxygen Species Is Independent of Tyrosine Kinase Receptor Phosphorylation and Requires Src Activity*

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Reactive oxygen species (ROS) participate as second messengers in the mitogenic signal transduction. Most of the experimental data supporting the role of ROS as signaling molecules have been obtained by using H2O2. Exposure of cells to H2O2 rapidly increases tyrosine phosphorylation of tyrosine kinase receptors (TKRs) in the absence of growth factor binding, thus inducing the activation of downstream signaling cascades, like that of protein kinase B (AKT). Another molecule able to induce an increase of intracellular ROS levels is diethylmaleate (DEM), which acts by depleting the ROS scavenger reduced glutathione (GSH). A comparison of the effects exerted by H2O2 and DEM shows that the latter induces redox modifications milder than those generated by H2O2. We also demonstrated that DEM-induced redox modifications are not accompanied by platelet-derived growth factor-receptor (PDGF-R) and epidermal growth factor-receptor Tyr phosphorylation, although they are able to activate ERKs and AKT, with kinetics different from those observed following H2O2 treatment. The activation of these two pathways is not blocked by AG1296, a selective inhibitor of PDGF-R Tyr kinase, thus confirming that the effects of DEM are not mediated by the TKR phosphorylation. On the contrary, PP2 (4-amino-5-(4-chlorophenyl)-7-(t-buty1)pyrazole[3,4-d]pyrimidine), an inhibitor of Src kinase, completely prevents DEM- and H2O2-induced AKT activation but has no effect on the pathway of ERKs. Finally, nitration of Tyr residues in PDGF-R is observed in DEM-treated cells, thus suggesting that ROS-induced modifications different from Tyr phosphorylation can occur at the growth factor-receptor level and can be involved in the regulation of signaling pathways.

A number of results demonstrate the involvement of ROS1 as a second messengers in signal transduction (1–7). A first group of data shows that one of the events triggered by the binding of growth factors (GF) and cytokines to their cognate receptors is the accumulation of ROS (8, 9). This phenomenon appears to play a major role in the transduction of the signal, considering that the inhibition of this accumulation, due to scavenging enzymes or to chemical antioxidants, prevents the activation of the downstream transduction pathways (10–12). Further support for the hypothesis of an involvement of ROS in signal transduction came from a second group of observations showing that the exposure of cultured cells to H2O2 results in Tyr phosphorylation of several growth factor receptors, in the absence of growth factor stimulation (1, 13). Tyr phosphorylation of GF-R seems to be strong enough to induce the activation of a downstream cascade of events, including AKT and MAPK activation, similar to that observed upon GF stimulation (14, 15). MAPK activation induced by ROS is also responsible for the accumulation of p21WAF1 (16, 17). Although a transient p21WAF1 accumulation is also observed upon GF stimulation, the induction of this protein by ROS is responsible, at least in part, for the growth arrest that is caused by ROS (16). ROS are also able to activate JNK and p38 (18): the first event is dependent on the activation of Src and, in turn, on Tyr phosphorylation of Cas, thus suggesting a physiological role for ROS in the transduction of signals involved in cell migration. On the contrary, p38 is not involved in the activation of JNK by ROS, as demonstrated by using PP2, the specific Src family Tyr kinase inhibitor (18).

Most of the mentioned experiments have been performed by exposing cells to different concentrations of H2O2, which is not a reactive oxygen species but is rapidly transformed in the hydroxyl radical (HO·), the most harmful ROS. It is still unclear which of the one or more ROS function as second messenger(s) in the cell, and it cannot be excluded that different types and/or concentrations of ROS could yield different effects on different targets within specific cell types. Furthermore, exogenous administration of H2O2, often at concentrations significantly higher than those even reached within the cell in phys-

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This manuscript is dedicated to the memory of Eraldo Antonini, eminent biochemist, prematurely deceased twenty years ago, on March 19th, 1983.

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1 The abbreviations used are: ROS, reactive oxygen species; GF, growth factor; AKT, protein kinase B; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH2-terminal kinase; DEM, diethylmaleate; GSH, reduced glutathione; GF-R, growth factor receptor; PDGF-R, platelet-derived growth factor receptor; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; DEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; TK, tyrosine kinase; TRK, TK receptor; PDGF-BB, platelet-derived growth factor BB; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-buty1)pyrazole[3,4-d]pyrimidine; PP3, 4-amino-7-phenylpyrazole[3,4-d]pyrimidine; eNOS, endothelial nitric-oxide synthase.
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**EXPERIMENTAL PROCEDURES**

**Materials and Cell Lines**—DEM, H₂O₂, lucigenin, aprotinin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, sodium fluoride, sodium orthovanadate, sodium pyrophosphate, and spermine NNOaote were from Sigma. The Comet assay was from Trevigen. The PDGF-Rβ-specific inhibitor AG1296, genistein, and the selective Src-inhibitor PP2 and its inactive analogue (PP3) were from Calbiochem; PDGF-βB, EGF, hydrcortisone, and insulin were from Upstate Biotechnology; anti-phospho ERKs, tubulin, and PDGF-Rβ antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-phosphotyrosine, anti-nitrotyrosine, and EGF-R antibodies were from Upstate Biotechnology. Anti-phospho AKT antibody was from New England BioLabs. Anti-eNOS antibody was from Oncogene. Anti-eNOS antibody was from Transduction Laboratories, and the anti-phospho eNOS antibody was from Cell Signaling Technology. Protein A (horseradish peroxidase-linked), anti-mouse IgG horseradish peroxidase, and protein A-Sepharose were from Amersham Biosciences.

Rat-2 fibroblasts and MCF10A were from ATCC. HEK293 cells stably transfected with eNOS were kindly provided by W. C. Sessa (21). Rat-2 fibroblasts and HEK293 were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5 and 10% fetal calf serum (FCS, HyClone), respectively, 100 units/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO₂/95% air at 37 °C. MCF10A were grown in a solution containing 50% DMEM and 50% Hank’s nutrient mixture F-12 (Euroclone), supplemented with 5% FCS (HyClone), 0.24 unit/ml insulin, 0.5 μg/ml hydrocortisone, 10 ng/ml EGF, 100 units/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO₂/95% air at 37 °C.

**Cell Culture Conditions and Treatments**—Cells were grown until they reached 90% confluence and then were starved in DMEM containing 0.2% FCS (Rat-2) or 1 mg/ml bovine serum albumin (MCF10A). After 48 h for Rat-2 and 84 h for MCF10A, cells were stimulated with DEM for 30 min, or with 1 mM H₂O₂ for 30 min or 1 mM DEM for 30 min, or in the absence or in presence of serum for the last 5 min. C and D, Rat-2 fibroblasts were grown in serum-free medium for 48 h (Starved) and then stimulated with 1 mM H₂O₂ for 30 min, or treated with 1 mM H₂O₂ for 30 min or 1 Mm DEM for 30 min, in the presence or in presence of the growth factor for the last 5 min.

**Western Blot and Immunoprecipitation Analyses**—Growth-arrested fibroblasts were treated with or without 20% FCS in the presence or absence of the appropriate amounts of DEM and H₂O₂ with or without tyrosine kinase inhibitors (see legends of figures for details) for the indicated times at 37 °C. Cells were rinsed with phosphate-buffered saline buffer (150 mM NaCl, 0.1 mM phosphate, pH 7.5) and then harvested in the same buffer. Cell lysates and Western blot analysis were performed as previously described (22). Antibodies were from Transduction Laboratories and Cell Signaling Technology. Western blotting was performed using 1 μg/ml anti-phosphotyrosine mouse monoclonal or anti-nitrotyrosine rabbit polyclonal IgG antibodies. Sre activity was assayed in Sre immunoprecipitates from oxidant-treated and untreated cells, using enolase as substrate (23).

**Lucigenin Assays**—To measure intracellular superoxide anion production we used lucigenin-enhanced chemiluminescence (10, 24) with slight modifications: exponentially growing Rat-2 cells were grown as above described and trypsinized; cell suspension was exposed to DEM and H₂O₂ for the times indicated in Fig. 4, centrifuged and suspended in Hank’s balanced salt solution (5.5 mM Na-glucose, 5.4 mM KCl, 0.44 mM KH₂PO₄, 136 mM NaCl, 4.2 mM NaHCO₃, 0.34 mM Na₃HPO₄) containing 1 mM lucigenin. Chemiluminescence was recorded using a Berthold LB 9505 lumimeter, and antibodies specific for phospho-ERKs and tubulin were from Cell Signaling Technology. Data are reported as the ratio between tail/mean + S.D. of four experiments was used to plot the data.

**Comet Assay**—DNA damages were analyzed using the Comet assay (25). Slight modifications to the manufacturer’s instructions were made: 1) the cells underwent the oxidizing treatments, as described in the figure legends, and were washed with PBS, trypsinized, re-suspended in PBS, and combined with LM-agarose (supplied in the Trevigen kit assay) at a ratio of 1:8 (cells:agarose). Electrophoretic run and qualitative and/or quantitative analyses were carried out according the Trevigen protocol. Quantitative analyses of the results were done by using the Image software (National Institutes of Health), as suggested by the manufacturer. Data are reported as the ratio between tail/nucleus areas on a log scale.

**Measurement of Nitrites**—After the oxidizing treatments, as described in the figure legends, the culture medium of Rat-2 and HEK293/eNOS cells was collected for the measurement of nitrites. Nitrites were assayed fluorometrically in microtiter plates using a standard curve of sodium nitrite (26).

**RESULTS**

Effects of Redox Changes Mediated by DEM and H₂O₂—Several experimental results indicate that signal transduction mediated by growth factors is accompanied by transient accumulation of ROS and that, in the absence of growth factors, exposure of cells to H₂O₂ mimics some molecular events that follow mitogenic stimulation, including Tyr phosphorylation of growth factor receptors (GF-Rs) and the activation of downstream cascades (1, 11). Diethylmaleate (DEM) is an indirect oxidant that yields an accumulation of endogenously produced ROS through a GSH depletion (20). An activation of ERK1 and ERK2 has been previously demonstrated following both H₂O₂
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Fig. 2. DEM is unable to induce PDGF and EGF receptor phosphorylation. Cell lysates containing 1 mg of protein were immunoprecipitated with antibodies specific for PDGF-R and EGF-R and subjected to immunoblot analysis with anti-phosphotyrosine antibodies (p-Tyr). The same filters were re-probed with anti-PDGF-R (A and B) or EGF-R (C and D) antibodies for the normalization of cell lysates. A and B, Rat-2 fibroblasts were treated as described in the legend for Fig. 1. After treatments (80 ng/ml PDGF-BB for 5 min, 1 mM H2O2 for 30 min, or 1 mM DEM for 30 min, in the absence or in presence of the growth factor for the last 5 min), the protein extracts were immunoprecipitated with antibodies specific for the PDGF-R β receptor. The arrows indicate the PDGF-R phosphorylated in Tyr (p-Tyr) or the total receptor present in the cell lysates. C and D, MCF10A cells were cultured as described under “Experimental Procedures,” serum-deprived for 84 h, stimulated with EGF (80 ng/ml for 5 min), or treated with 1 mM H2O2 for 30 min or DEM for 30 min, in the absence or in presence of the growth factor for the last 5 min. The arrows indicate the EGFR phosphorylated in Tyr (p-Tyr) or the total receptor present in the cell lysates.

One of the mechanisms responsible for the observed effects is MAPK activation and the phosphorylation of AKT. The exposure of cells to H2O2 mimics the effects of growth factors even in the absence of mitogenic stimulation, whereas treatment with DEM is unable to change phospho-Tyr content of these two proteins, both in the presence or in the absence of growth factor stimulation.

Protein kinase B (AKT) is another target of ROS downstream the TKR (27). We therefore studied the effects of DEM treatment on the activation of this Ser-Thr protein kinase and observed a further difference by comparing the effects of H2O2 and DEM on the phosphorylation of AKT. Fig. 3 shows a Western blot of total cell lysates from starved Rat-2 fibroblasts stimulated with serum (panels A and B) or with PDGF-BB (panels C and D). An increased phosphorylation of AKT can be observed using antibodies specific for the phosphorylated Ser-473 of the kinase. A similar activation can be induced by the treatment with H2O2, as well as by the two concomitant treatments (mitogenic and oxidant). However, the effects of DEM on the activation of AKT appear to be different: as shown in Fig. 3B, the phosphorylation of this kinase in cells exposed only to DEM is very low, much lower in comparison to H2O2 treatment, but when serum and DEM are added simultaneously, these two treatments yield a strong synergistic effect on AKT phosphorylation at Ser-473. This result is less pronounced when PDGF-BB, instead of serum, is used to stimulate starved fibroblasts (Fig. 3D). The explanation for this could probably be based on the higher extent of AKT activation by the purified growth factor.

One of the mechanisms responsible for the observed effects could be related to possible qualitative and/or quantitative differences in the ROS produced by the two oxidants: to this aim, we exposed Rat-2 fibroblasts to DEM and to H2O2 and measured the generation of superoxide anion using the lucigenin assay (10, 24). As shown in Fig. 4, when Rat-2 fibroblasts are exposed to DEM, a peak of superoxide anion production is observed 30 min after DEM treatment (see Fig. 4A), which follows the DEM-induced GSH depletion (data not shown), whereas, upon treatment of cells with H2O2, the production of superoxide ion is much faster, reaching the maximal value...
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Fig. 4. Kinetics of ERKs and AKT activation by H2O2 and DEM. A, production of superoxide anion during treatment with DEM (upper panel) and H2O2 (lower panel). Rat-2 fibroblasts were grown as described under “Experimental Procedures” and trypsinized, and the cell suspension was exposed to DEM and H2O2 (1 mM) for the times indicated. Chemiluminescence was recorded as described under “Experimental Procedures.” RLU, relative lucigenin-enhanced chemiluminescence units. Data are representative of four independent experiments. B, time-course experiments of ERKs and AKT activation by DEM and H2O2. Rat-2 fibroblasts, grown in serum-free medium for 48 h, were treated with 1 mM DEM (upper panel) or with 1 mM H2O2 (lower panel) for the times indicated. Cell lysates containing 25 μg of proteins were analyzed by electrophoresis as described in the legend for Fig. 1 and subjected to immunoblot analysis with antibodies specific for phospho-ERKs (p-ERKs) or phospho-AKT (p-AKT). The same filters were re-probed with anti-tubulin antibodies for the normalization of cell lysates. The arrows indicate the phosphorylated forms of ERKs and AKT, respectively, and the tubulin. C, time-course experiments of PDGF-Rβ activation by DEM. Rat-2 fibroblasts, grown in serum-free medium for 48 h, were treated with 1 mM DEM for the times indicated. Cell lysates containing 1 mg of proteins were immunoprecipitated with antibodies specific for PDGF-Rβ and subjected to immunoblot analysis with anti-phosphotyrosine antibodies (p-Tyr) as described in the legend for Fig. 2. The same filters were re-probed with anti-PDGFR-β antibodies for the normalization of cell lysates.

After 1 min, and the amount of ROS generated by H2O2 treatment is at least 10 times higher (Fig. 4A). The differences between DEM and H2O2 suggest the hypothesis that also the activation of MAPK and AKT by these two oxidizing agents could be different. To evaluate this phenomenon we performed time course experiments of MAPK and AKT activation during the treatment with both oxidizing agents: as shown in Fig. 4B, the peak of ERKs and AKT phosphorylation following the treatment with H2O2 is observed already 1–5 min after the treatment, whereas a longer time (30 min) is required for DEM. However, the delay of DEM-induced ERKs activation is not sufficient to explain the absence of TKR phosphorylation, because, even at a longer time of DEM exposure (up to 3 h), the phosphorylation of PDGF-R was undistinguishable from that of starved cells (Fig. 3C). One possible explanation of the observed differences between H2O2 and DEM effects could be the different levels of ROS generation following the exposure of cells to the two agents. In the attempt to analyze H2O2 and DEM effects in comparable experimental conditions, we measured Tyr phosphorylation of PDGF-R following the treatment of cells with various concentrations of H2O2 and DEM. At the same concentration the extent of DNA damage provoked by ROS was measured by using the comet assay (24). As shown in Fig. 5, even at a comparable degree of DNA damage provoked by DEM and H2O2, as for example 0.05 mM H2O2 and 1 mM DEM (see Fig. 5A), the PDGF-R Tyr phosphorylation is induced only by the treatment with H2O2 (see Fig. 5B).

Activation of a Non-receptor Tyrosine Kinase Is Involved in DEM-induced Effects—To investigate on possible mechanisms involved in the activation of mitogenic cascade in the absence of the activated tyrosine kinase receptor, we exposed Rat-2 fibroblasts to genistein, a competitive inhibitor of ATP binding to the catalytic domain of tyrosine kinase and inhibitor of tyrosine kinase activity of both growth factor receptor and non-receptor Tyr kinases. As shown in Fig. 6A, the activation of AKT by DEM is blocked by genistein pretreatment. To test the involvement of a non-receptor tyrosine kinase in the activation of AKT following DEM treatment, we focused our attention on Src, a tyrosine kinase activated by H2O2 (28). To this aim we exposed Rat-2 cells to DEM and H2O2 and assayed Src tyrosine kinase activity on a heterologous substrate: Fig. 6B shows an increased Src activity, measured through the phosphorylation of the enolase, in Src immunoprecipitates from DEM- and H2O2-treated cells. The involvement of Src in the activation of AKT was confirmed by exposing the cells to PP2, a selective Src inhibitor, and to PP3, a structurally inactive analogue. Fig. 6C shows a specific inhibition of DEM- and H2O2-induced AKT activation in the presence of this Src inhibitor. On the contrary, PP2 treatment was ineffective in the abolishment of MAPK activation by DEM (Fig. 6C); this last finding is in agreement with previously described results demonstrating that H2O2-induced ERK1 and p38 activities in endothelial cells are Src-independent (18). These results are consistent with a Src-mediated activation of AKT upon oxidizing treatments.

DEM-mediated Effects on AKT and ERKs Are Independent of Tyrosine Kinase Receptor Phosphorylation—Fig. 7A illustrates a Western blot of total cell lysates from starved Rat-2 fibroblasts exposed to PDGF-BB, DEM, or H2O2, with or without AG1296, a specific inhibitor of the tyrosine kinase activity of PDGF-R. The effects of DEM on AKT do not require PDGF-R phosphorylation: in fact, Fig. 7A shows that, although the activation of AKT by PDGF-BB is highly decreased in Rat-2 cells pretreated with the inhibitor, the same treatment does not yield any effect in cells exposed to DEM. Surprisingly, in cells pretreated with the PDGF-R inhibitor and then exposed simultaneously to the growth factor and to DEM, the effect of DEM is reinforced. Similar results were obtained for ERKs. As a control, the analysis of Tyr phosphorylation of PDGF-R is
shown to exclude direct inactivation of AG1296 inhibitor by the oxidizing treatments (Fig. 7A). These results confirm that the activation of downstream targets following DEM treatment does not require tyrosine autophosphorylation of PDGF-R but suggest that PDGF-PDGF-R interaction could have some role in the activation of downstream cascades, even in the absence of receptor phosphorylation. We therefore hypothesized that modifications of Tyr residues other than phosphorylation could be involved in the activation of the downstream cascade: to this aim we focused our attention on tyrosine nitration, a modification that can occur in proteins and is involved in the modulation of signaling processes starting from receptors of tyrosine kinases to downstream signaling cascades (29, 30). Fig. 7B shows a Western blot of cell lysates from starved Rat-2 fibroblasts exposed to serum, DEM, or spermine NONoate, a positive control of intracellular NO generation. After these treatments cells were immunoprecipitated with anti-PDGF-R antibodies and then analyzed by immunoblot with anti-nitrotyrosine antibody: the amount of PDGF-R tyrosine nitration in DEM-treated cells was comparable to that present in the positive control, whereas it seemed to be absent in serum-treated cells. To investigate how DEM treatment induces the nitrosylation of PDGF-R, we measured the amount of NO produced by Rat-2 cells after DEM treatment. A total nitrites assay in the culture medium demonstrated that a significant nitrite accumulation was present after DEM exposure. Conversely, in the same experimental conditions, treatment with H2O2 was ineffective (Fig. 8A). Furthermore, we have also demonstrated that DEM-induced NO accumulation is due to eNOS activation: this specific matter was demonstrated by using HEK293 cells stably transfected with eNOS and exposed to the two oxidizing agents (Fig. 8A). Interestingly, by using antibodies specific for the phosphorylated Ser-1177 of the endothelial nitric-oxide synthase, we demonstrated that DEM treatment promotes eNOS phosphorylation, which indicates eNOS activation (Fig. 8B).

**DISCUSSION**

A number of results have accumulated in the literature on the involvement of ROS in growth factor signal transduction.
Most of these data have been obtained upon the exposure of cells to H\textsubscript{2}O\textsubscript{2}, which causes tyrosine kinase receptor phosphorylation, in the absence of growth factor stimulation. In this report we have demonstrated that redox modifications induced by DEM are accompanied by the activation of ERKs and AKT, even if TKR phosphorylation is completely absent.

This different behavior between DEM and H\textsubscript{2}O\textsubscript{2} could be due to qualitative and/or quantitative differences in the ROS generated by these two agents. In fact, the timing and the extent of superoxide anion production are very different, being much higher, at least 10 times compared with DEM, and faster in the cells treated with H\textsubscript{2}O\textsubscript{2}. Furthermore, we found that DEM induces milder redox modifications, as demonstrated by the lower extent of DNA damage in cells exposed to this compound, compared to that observed in H\textsubscript{2}O\textsubscript{2}-treated cells. The timing and the quantitative differences of ROS production could explain the different timing of MAPK and AKT activation by DEM and H\textsubscript{2}O\textsubscript{2}; in fact, both kinases are rapidly activated by H\textsubscript{2}O\textsubscript{2} (within 1 min), whereas longer exposures to DEM are required. However, even by using concentrations of H\textsubscript{2}O\textsubscript{2} and DEM leading to similar intracellular redox modifications, Tyr kinase inhibitor activity, given the abolishment of the phenomenon in cells treated with genistein. The control value was 0.09 ± 0.08 nM. B, DEM treatment induces eNOS phosphorylation in HEK293/eNOS cells. Growing HEK293/eNOS cells were treated with DEM 1 mM, H\textsubscript{2}O\textsubscript{2} 50 μM, and 500 nM sphingosine 1-phosphate (SP) for 30 minutes. Cell lysates containing 35 μg of protein were analyzed by Western blot anti-phospho eNOS (p-eNOS); the same filter was re-probed with an antibody anti-eNOS for the normalization of cell lysates. The arrows indicate the phosphorylated forms of eNOS (p-eNOS) and the total eNOS, respectively.

The non-receptor Tyr kinase Src is activated by both DEM and H\textsubscript{2}O\textsubscript{2}, and treatment of cells with a specific inhibitor of this kinase, PP2, completely blocks AKT activation by both pro-oxidant molecules. These results support the hypothesis that intracellular ROS accumulation leads to the activation of non-receptor TKs, without a contemporary phosphorylation of GF-R. This phenomenon triggers the induction of AKT survival pathway and has no effect on the machinery of ERKs. This last result is in agreement with the data of Yoshizumi et al. (18) demonstrating that neither ERK or p38 are activated by H\textsubscript{2}O\textsubscript{2} through an Src-dependent mechanism.
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To explain these observations a direct effect of ROS on Src TK activity could be hypothesized, although it is quite surprising that ROS selectively activate Src and not GF-R. As mentioned above, one possibility is that ROS generated by DEM are restricted to the intracellular compartments where they are normally generated (e.g. mitochondria); it is possible that in these areas they are only challenged with non-receptor TK as Src and not with membrane bound GF-R. The mechanisms through which ERKs are activated by DEM in the absence of both GF-R and non-receptor TK remain to be established. Previous results demonstrated that this activation of ERKs is blocked by dominant negative mutants of ras (17), and this result contributes to the positioning of ROS targets on ras itself or upstream of this molecule.

The results obtained with the selective PDGF-R inhibitor AG1296, besides being of support for a PDGF-R-phospho-Tyr-independent activation of AKT and ERKs upon DEM treatment, also suggest an interesting hypothesis. In fact, even though PDGF-R is not phosphorylated following DEM treatment, in the presence of the specific PDGF-R inhibitor AG1296 and the growth factor, the effects of DEM are significantly reinforced. These results suggest that the PDGF-PDGF-R complex could have some role in the activation of downstream cascades, even in the absence of receptor autophosphorylation, e.g. PDGF-PDGF-R could act as a scaffold protein, facilitating the transduction of the signals independently from its Tyr kinase activity. Other proteins interacting with tyrosine kinase receptors not through TKR tyrosine phosphorylation include the Enigma protein whose binding to Ret/ptc2, the constitutively active, oncogenic form of the c-Ret receptor tyrosine kinase, in the presence of the specific PDGF-R inhibitor AG1296, besides being of support for a PDGF-R phospho-Tyr-independent activation of AKT and ERKs upon DEM treatment; both processes can converge on and contribute to AKT activation.

In conclusion, our results demonstrate that ROS can activate signaling cascades, even in the absence of growth factor receptor phosphorylation, and suggest the possibility of a new mechanism for the activation of transduction pathways based on Tyr nitration of TKRs. From a regulatory point of view it should be ascertained that, due to the existence of specific “denitrases” (38), the nitration of Tyr residues is a reversible process.

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