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The Raf/MEK inhibitor PD98059 enhances ERK1/2 phosphorylation mediated by peroxynitrite via enforced mitochondrial formation of reactive oxygen species

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Abstract Exposure of PC12 cells to 100 μM peroxynitrite promotes phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) sensitive to PD98059 or U0126. At higher concentrations, however, ERK1/2 phosphorylation was prevented by U0126 and increased by PD98059 via a U0126-sensitive mechanism. PD98059, unlike U0126, enhanced the peroxynitrite-dependent formation of reactive oxygen species (ROS). These results, along with others obtained using respiratory chain inhibitors and respiration-deficient cells, lead to the conclusion that PD98059, while effectively inhibiting the peroxynitrite-induced Raf/MEK signaling leading to ERK1/2 phosphorylation, promotes an enforced mitochondrial formation of ROS inducing ERK1/2 phosphorylation via a Raf-1-independent/MEK-dependent mechanism.

Key words: Peroxynitrite; PD98059; Extracellular signal-regulated kinase phosphorylation; Reactive oxygen species; Mitochondrion; PC12 cell

1. Introduction

Peroxynitrite, a powerful biological oxidant, effectively stimulates the phosphorylation of the extracellular signal-regulated kinase (ERK) isoforms p44 (ERK1) and p42 (ERK2) in different cell types [1–4]. In rat pulmonary myofibroblasts peroxynitrite, as well as H_2O_2, activates the epidermal growth factor receptor (EGRF), Raf-1 and mitogen-activated protein kinase kinase (MEK) but, unlike H_2O_2, induces these effects independently so that ERK phosphorylation was also observed when the EGRF tyrosine kinase or Raf-1 kinase was blocked [2]. Zouki et al. [4] found that peroxynitrite activates ERK via the Ras/Raf/MEK signaling cascade also in human neutrophils. A different study [3] showed that the peroxynitrite-dependent activation of ERK1/2 in rat-1 fibroblasts is mediated by protein kinase C (most likely a calcium-dependent isoform) in a MEK-independent manner. Thus, it appears that different pathways are involved in the activation of ERK1/2 phosphorylation induced by peroxynitrite in different cell types. Not surprisingly, PD98059 inhibited ERK1/2 phosphorylation induced by peroxynitrite in rat pulmonary myofibroblasts [2] or human neutrophils [4] but was ineffective in rat-1 fibroblasts [3].

We herein report experimental evidence of a novel effect of PD98059 in cells exposed to peroxynitrite. In PC12 cells, the oxidant appears to stimulate ERK1/2 phosphorylation via a MEK-dependent mechanism that can be prevented by PD98059. Under the same conditions, however, the inhibitor greatly enhances the peroxynitrite-dependent mitochondrial formation of reactive oxygen species (ROS) promoting ERK1/2 phosphorylation via a MEK-dependent, Raf-1-independent mechanism. Thus, under conditions of combined exposure to PD98059 and peroxynitrite the extent of ERK1/2 phosphorylation is much greater than that mediated by peroxynitrite alone.

2. Materials and methods

2.1. Cell culture and treatments

PC12 rat pheochromocytoma respiration-proficient and -deficient cells were cultured as previously described [5]. For experiments, cells (2.5×10^5) were inoculated into 60 mm tissue culture dishes and grown for 18–24 h. At the treatment stage, total cell number was between 4.0 and 4.5×10^5 cells/dish. Treatments were performed in 2 ml of saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l $NaHCO_3$ and 0.9 g/l glucose) at 37°C. Peroxynitrite was synthesized as previously described [6] and added to the cultures as a bolus. PD98059 and U0126 (Alexis Biochemicals, Florence, Italy) were dissolved in dimethyl sulfoxide; at the treatment stage the final concentration of dimethyl sulfoxide was never higher than 0.05%.

2.2. Western blot analysis

Following treatments, cells were washed twice with phosphate-buffered saline, scraped into ice-cold lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate and 1 mM sodium fluoride, pH 8.0) and incubated on ice for 1 h. Cells were then sonicated and centrifuged for 10 min at $21\,500\times g$ (4°C). Supernatants were assayed for protein concentration using Bio-Rad protein assay reagent. Protein samples (25 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and electrotransferred to polyvinylidene difluoride membranes. The blots were blocked for 1 h at room temperature with 5% milk powder in Tris-buffered saline (140 mM NaCl, 50 mM Tris–HCl, pH 7.2) containing 0.06% Tween 20 and probed with a primary antibody against phospho-ERK1/2 (1:700) or ERK1/2 (1:1000) overnight at 4°C. Horseradish peroxidase-conjugated monoclonal or polyclonal antibodies (1:2000) were used for enhanced chemiluminescence detection. The antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Densitometric analysis of blots was performed using the electrophoresis documentation and the public domain NIH Image

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Abbreviations: ERK1/2, extracellular signal-regulated kinases 1 and 2; EGRF, epidermal growth factor receptor; MEK, MAPK/ERK kinase; ROS, reactive oxygen species; FBS, fetal bovine serum; DHR, dihydrorhodamine 123

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1.62 program (developed at the US National Institutes of Health and available on Internet at http://rsb.info.nih.gov/nih-image/).

2.3. Oxidation of dihydrorhodamine

The cells were treated and then loaded with 10 μM dihydrorhodamine 123 (DHR, Molecular Probes Europe, Leiden, The Netherlands) for 10 min in saline A containing 5% fetal bovine serum (FBS) at 37°C. After accurate washes cellular fluorescence was imaged and quantified as previously described [5].

2.4. Statistical analysis

The results are expressed as means ± S.E.M.; statistical analysis of the data for multiple comparisons was performed by the analysis of variance followed by Dunnett's test.

3. Results and discussion

In keeping with previous findings from other laboratories, exposure of PC12 cells to KCl (75 μM, [7]), FBS (20% [8]), H_2O_2 (1 mM [9]) or peroxynitrite (500 μM [1]) resulted in increased ERK1/2 phosphorylation, whereas no change in the amount of ERK proteins was observed (Fig. 1A,B). Phos-

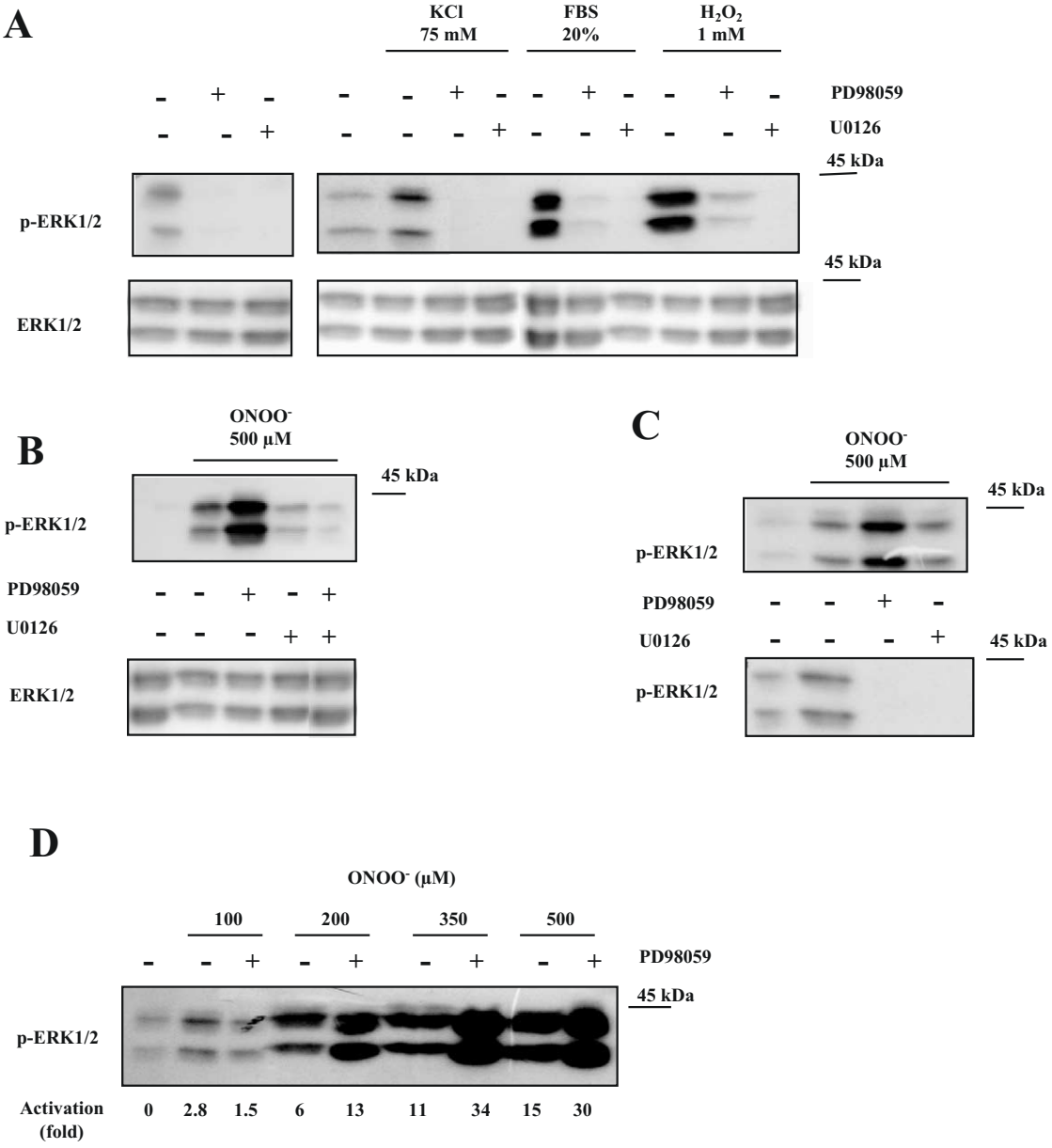

Fig. 1. The effect of PD98059 or U0126 on ERK1/2 phosphorylation induced by peroxynitrite and other stimuli. A: Cells were exposed for 10 min to 50 μM PD98059 or 20 μM U0126, and subsequently treated with 75 mM KCl (5 min), 20% FBS (10 min) or 1 mM H_2O_2 (10 min). Cell were then lysed for Western blot analysis using antibodies recognizing phospho- and total ERK proteins. B: Cells pre-exposed (10 min) to the ERK inhibitors (see above) and treated for a further 15 min with 500 μM peroxynitrite were analyzed as detailed in A. C: Cells pre-exposed (10 min) to the ERK inhibitors and then treated with 500 μM peroxynitrite for 15 min in fresh saline in the absence of the ERK inhibitors were processed for Western blot analysis using phospho-ERK antibodies (top). The blot shown at the bottom reports the results of an experiment in which the cells were treated for 5 min with 500 μM peroxynitrite and then post-incubated for a further 10 min in the presence of either PD98059 or U0126. D: Cells pre-exposed to PD98059 for 10 min were treated for an additional 15 min with increasing concentrations of peroxynitrite and then analyzed as detailed in C. Fold increase in ERK1/2 activation is shown below each lane of the blot. Each of the blots shown above (A–D) is representative of at least five experiments with similar outcomes.

phorylation of ERK1/2 induced by the above different stimuli seems to require MEK since this response was abolished by U0126 (20 μM), which inhibits MEK activation and activity [10]. Surprisingly, however, PD98059 (50 μM), which binds directly to the non-phosphorylated form of MEK after blocking its activation by Raf-1 [10], markedly reduced ERK1/2 phosphorylation induced by KCl, FBS and H_2O_2 but actually enhanced phosphorylation induced by peroxynitrite. Importantly, U0126 suppressed ERK1/2 phosphorylation induced by the cocktail PD98059/peroxynitrite (Fig. 1B).

Thus, these results on the one hand confirm that KCl, FBS and H_2O_2 activate ERK1/2 and that U0126 more potently than PD98059 inhibits these responses [10] whereas, on the other hand, they provide unexpected experimental evidence suggesting that PD98059 does not inhibit but, rather, enhances the peroxynitrite-induced ERK1/2 phosphorylation. The observation that the latter response is sensitive to U0126 is consistent with an involvement of MEK, which must be activated via a Raf-1-independent mechanism. These results were reproducibly obtained using 10 different preparations of peroxynitrite and four lots of PD98059. Furthermore, decomposed peroxynitrite, or the vehicle, failed to stimulate ERK1/2 phosphorylation produced by peroxynitrite both in the absence and in the presence of PD98059 (not shown). It is important to note that peroxynitrite was used at 500 μM, a condition failing to produce obvious signs of toxicity detectable by visual inspection or by the trypan blue exclusion/lactate dehydrogenase assays (not shown). Other studies investigating ERK1/2 phosphorylation employed similar, or higher, concentrations of peroxynitrite [1,2].

In an attempt to further characterize the effects of PD98059, we performed experiments in which the cells were incubated for 10 min with the inhibitor and then, after accurate washing, exposed for a further 15 min to peroxynitrite. As illustrated in Fig. 1C (top) this regimen also caused enhanced ERK1/2 phosphorylation. However, PD98059 added to the cultures 5 min after peroxynitrite, a time at which the oxidant is already decomposed [11], caused inhibition of phosphorylation (Fig. 1C, bottom). Thus PD98059, in order to mediate enhanced ERK1/2 phosphorylation, must be within the cells at the time of peroxynitrite exposure. An additional conclusion from these experiments is that peroxynitrite, consistently with previously published data [2,4], promotes ERK1/2 phosphorylation via a Raf-1-dependent mechanism. We next investigated the effect of increasing concentrations of peroxynitrite and found that ERK1/2 phosphorylation was clearly detectable at 100 μM and dose-dependent up to 500 μM (Fig. 1D). Interestingly, the enhancing effects of PD98059 were noted when this treatment was combined with peroxynitrite levels greater than 100 μM whereas, at this concentration, inhibition was rather observed. We interpret these findings as an indication that concentrations of peroxynitrite higher than 100 μM are required to promote, when associated with PD98059, a Raf-1-independent ERK1/2 phosphorylation.

The fluorescent probe DHR, which accumulates in the mitochondria and fluoresces when oxidized by various species, was utilized to obtain biochemical evidence of delayed ROS formation in PC12 cells exposed to peroxynitrite. In these experiments, the cells were treated with 500 μM peroxynitrite for 5 min, exposed for an additional 10 min to 10 μM DHR and finally examined by confocal microscopy. Under these conditions, peroxynitrite, which readily decomposes after its addition to the cultures [11], cannot directly oxidize DHR. The oxidant caused a slight, although statistically significant, increase in the fluorescence response (Fig. 2A) sensitive to the peroxynitrite scavengers L-methionine (20 mM) and Trolox (1 mM) (not shown). Pre-exposure to U0126 did not affect basal fluorescence or the ability of peroxynitrite to promote oxidation of DHR. In contrast, not only did PD98059 generate a fluorescence response in the absence of additional treatments but, most importantly, it dramatically increased the delayed oxidation of DHR mediated by peroxynitrite. It is unclear whether the effect of PD98059 alone is due to formation of ROS or to the intrinsic fluorescence of the compound. Although further investigation is needed to address this issue, we favor the second possibility since time dependence studies indicated that oxidation of DHR remains stable after addition of the inhibitor but progressively increases upon supplementation with peroxynitrite (not shown). Thus, the ability of PD98059 to increase ROS formation after exposure to peroxynitrite may explain its effects on ERK1/2 phosphorylation, which are remarkably different from those mediated by U0126. It is indeed well established that ROS are effective inducers of ERK1/2 phosphorylation [12].

Peroxynitrite promotes important effects at the level of the mitochondrial respiratory chain and, among these, we previously identified delayed formation of superoxides and H_2O_2 as a consequence of the peroxynitrite-dependent inhibition of complex III [13]. We reasoned that PD98059 might enhance this response thereby promoting a flux of ROS responsible for ERK1/2 phosphorylation. The results illustrated in Fig. 2B are consistent with this notion since the complex I inhibitor rotenone, at a concentration (0.5 μM) suppressing oxygen consumption [5,13], also caused inhibition of ERK1/2 phosphorylation mediated by PD98059/peroxynitrite but did not affect the response elicited by peroxynitrite alone. Similarly, rotenone failed to prevent ERK1/2 phosphorylation induced by FBS (Fig. 2C). Since rotenone suppressed delayed ROS formation mediated by peroxynitrite, both in the absence and in the presence of PD98059 (Fig. 2A), we conclude that mitochondrial ROS are involved in ERK1/2 phosphorylation induced by PD98059/peroxynitrite, but not by peroxynitrite alone.

Peroxynitrite effectively stimulates ERK1/2 phosphorylation also in respiration-deficient cells (Fig. 2D) and this response, while not affected by rotenone (not shown), is significantly reduced by U0126 as well as by PD98059. In these cells, peroxynitrite (with or without PD98059) failed to promote formation of superoxides or H_2O_2 (not shown) and, as a consequence, the notion that ROS are not involved in ERK1/2 phosphorylation mediated by peroxynitrite is further established. In addition, the observation that under conditions not permissive for ROS formation PD98059 inhibits ERK1/2 phosphorylation mediated by peroxynitrite on the one hand emphasizes the specificity of the effects of the MEK inhibitor and, on the other hand, demonstrates that a functional respiratory chain is required in order for peroxynitrite and PD98059 to promote formation of ROS responsible for ERK1/2 phosphorylation.

In conclusion, our findings indicate that peroxynitrite promotes PC12 cell ERK1/2 phosphorylation mediated by a Raf-MEK-dependent mechanism. Consistently, the response elicited by low peroxynitrite concentrations (100 μM) was inhib-

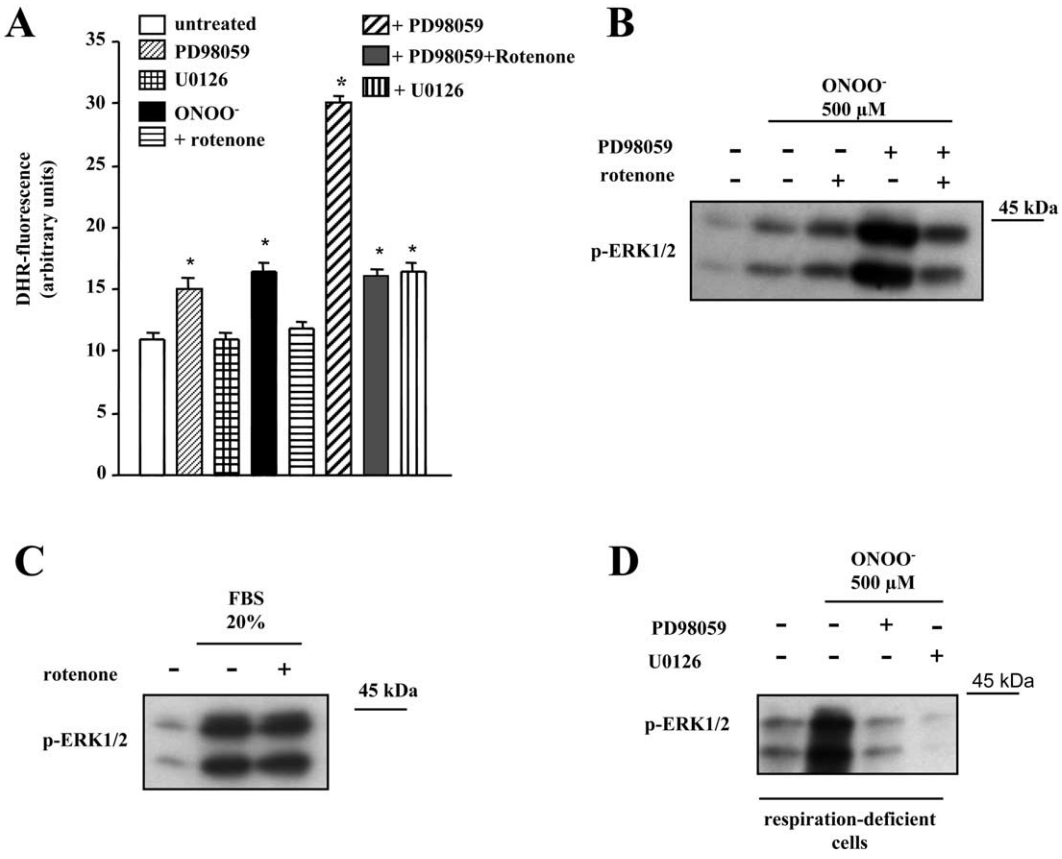

Fig. 2. ERK1/2 phosphorylation induced by PD98059 in cells exposed to peroxynitrite is dependent on enforced mitochondrial formation of ROS. A: The bar graph shows the quantitative analysis of the DHR fluorescence response mediated by 500 μM peroxynitrite in the absence or presence of PD98059, U0126, rotenone (0.5 μM) or a combination of rotenone and PD98059. The inhibitors were given to the cells 10 min prior to peroxynitrite exposure. Results are expressed as arbitrary units and represent the mean ± S.E.M. calculated from three to five separate experiments, each performed in duplicate. $^{*}P < 0.01$ vs. untreated cells (analysis of variance followed by Dunnett's test). B: Cells were pre-exposed for 10 min to PD98059, rotenone or a combination of the two agents and then treated for an additional 15 min with peroxynitrite. The cells were then processed for Western blot analysis using phospho-ERK antibodies. C: Cells were pre-exposed for 10 min to rotenone, incubated for a further 10 min with 20% FBS and then analyzed as detailed in B. D: Respiration-deficient cells were pre-exposed for 10 min to PD98059 or U0126 and then treated for an additional 15 min with peroxynitrite. The cellular extracts were then analyzed as detailed in B. Each of the blots shown above (B–D) is representative of at least five experiments with similar outcomes.

ited by either U0126 or PD98059. PD98059 also inhibited the pathway leading to Raf/MEK/ERK1/2 phosphorylation triggered by higher concentrations of peroxynitrite but, under these conditions, promoted parallel events causing enforced mitochondrial formation of ROS responsible for the activation of a Raf-1-independent pathway resulting in MEK/ERK1/2 phosphorylation. In order to produce these effects, PD98059 has to be given to the cultures at the time of peroxynitrite exposure and acted as an inhibitor of ERK phosphorylation when added after peroxynitrite. Importantly, PD98059 inhibited ERK phosphorylation in respiration-deficient cells also under conditions of concomitant exposure with peroxynitrite.

As stated above, peroxynitrite was not toxic for the cells in the absence of additional treatments. The question therefore arises as to whether activation of ERK1/2 plays a role in survival and, if this is the case, then the above observations lead to the possibility that PD98059 and U0126 differentially impact on cell survival. Furthermore, it may be expected that PD98059 promotes different effects in cells exposed to 100 μM, or higher, concentrations of peroxynitrite. As a final note, our results indicate PD98059 should be carefully utilized as a MEK inhibitor, in particular under conditions involving exposure to either authentic peroxynitrite or agents resulting in the formation of endogenous peroxynitrite.

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