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Pleiotrophin-induced endothelial cell migration is regulated by xanthine oxidase-mediated generation of reactive oxygen species

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ABSTRACT

Pleiotrophin (PTN) is a heparin-binding growth factor that induces cell migration through binding to its receptor protein tyrosine phosphatase beta/zeta (RPTPβ/ζ) and integrin alpha v beta 3 ($\alpha_v\beta_3$). In the present work, we studied the effect of PTN on the generation of reactive oxygen species (ROS) in human endothelial cells and the involvement of ROS in PTN-induced cell migration. Exogenous PTN significantly increased ROS levels in a concentration and time-dependent manner in both human endothelial and prostate cancer cells, while knockdown of endogenous PTN expression in prostate cancer cells significantly down-regulated ROS production. Suppression of RPTPβ/ζ through genetic and pharmacological approaches, or inhibition of c-src kinase activity abolished PTN-induced ROS generation. A synthetic peptide that blocks PTN–$\alpha_v\beta_3$ interaction abolished PTN-induced ROS generation, suggesting that $\alpha_v\beta_3$ is also involved. The latter was confirmed in CHO cells that do not express β_3 or over-express wild-type β_3 or mutant β_3Y773F/Y785F. PTN increased ROS generation in cells expressing wild-type β_3 but not in cells not expressing or expressing mutant β_3. Phosphoinositide 3-kinase (PI3K) or Erk1/2 inhibition suppressed PTN-induced ROS production, suggesting that ROS production lays down-stream of PI3K or Erk1/2 activation by PTN. Finally, ROS scavenging and xanthine oxidase inhibition completely abolished both PTN-induced ROS generation and cell migration, while NADPH oxidase inhibition had no effect. Collectively, these data suggest that xanthine oxidase-mediated ROS production is required for PTN-induced cell migration through the cell membrane functional complex of $\alpha_v\beta_3$ and RPTPβ/ζ and activation of c-src, PI3K and ERK1/2 kinases.

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Introduction

Pleiotrophin (PTN) is a heparin binding growth factor with a significant regulatory role on inflammation, angiogenesis and cancer (Pantazaka and Papadimitriou, 2012; Papadimitriou et al., 2009; Silver et al., 2012; Yokoi et al., 2012; Zhang et al., 2013). In all cases, the best characterized PTN function is its effect on cell migration through its receptor protein tyrosine phosphatase beta/zeta (RPTPβ/ζ) (Lu et al., 2005; Polykratis et al., 2005) and integrin alpha v beta 3 ($\alpha_v\beta_3$) (Mikelis et al., 2009). Part of the downstream signaling has been elucidated and involves c-Src kinase, phosphoinositide 3-kinase (PI3K) and ERK1/2 activation (Koutsioumpa et al., 2013; Mikelis et al., 2009; Polykratis et al., 2005). However, a lot of work is still required in order to uncover more players, as well as to pinpoint how all these players communicate to regulate the effect of PTN on cell migration.

Reactive oxygen species (ROS), like hydrogen peroxide, superoxide and the hydroxyl radical, are constantly produced during normal metabolism and in response to external stimuli. At higher concentrations following oxidative stress situations, ROS may damage proteins, lipids and DNA, and may induce apoptosis. In contrast, after stimulation by various external factors, including growth factors, hormones and cytokines, or cellular events such as adhesion, cells will produce low local amounts of ROS (Dhar-Mascareno et al., 2003; Hatziapostolou et al., 2006; Terman et al., 1992), which have been identified as essential mediators that regulate the transduction of signals from the membrane to the nucleus via oxidation and reduction in proteins (Holmström and Finkel, 2014) and lead to angiogenesis stimulation (Kim and Byzova, 2014; Polytarchou and Papadimitriou, 2004, 2005, Polytarchou et al., 2009) and inflammation linked to several pathologies (Bryan et al., 2012; Naik and Dixit, 2011). ROS are produced by a number of pathways, including the mitochondrial electron transport chain, cytochrome P450, xanthine oxidase (XO), uncoupled nitric oxide synthase and the family of NADPH oxidase enzymes (Bir et al., 2012; Wilkinson-Berka et al., 2013).

The aim of the present study was to investigate whether PTN affects ROS production by human endothelial and prostate cancer cells and whether ROS production is involved in PTN-induced cell migration.

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Materials and methods

Materials

Human recombinant PTN was from PeproTech, Inc. (Rocky Hill, NJ, USA). The NAD(P)H oxidase-specific inhibitor 4-hydroxy-3-methoxyacetophenone (apocynin) and the ROS-sensitive fluorescent dye, 5(6)-carboxy-2,7-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) were purchased from Fluka. The hydrogen peroxide scavenger catalase, the NAD(P)H oxidase inhibitors 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF) and 3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo(4,5-d)pyrimidine (VAS2780), the XO inhibitor allopurinol and the general phosphatase inhibitor sodium orthovanadate were purchased from Sigma. The XO inhibitor febuxostat was from Santa Cruz Biotechnology, Inc. The mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitor U0126 and the src kinase inhibitor PP1 were purchased from Tocris (Ellisville, MO, USA). RNA oligonucleotide primers for RPTPβ/ζ (Polykratis et al., 2005) were obtained from VBCBiotech Services (Vienna, Austria), and for human XO were obtained from Santa Cruz Biotechnology, Inc. Double-stranded negative control siRNA was from Ambion (Austin, TX, USA). B3 (CYDMKTTC) and B3 scrambled (TKCMTCDY) peptides were from Cambridge Peptides (Birmingham, UK). All the inhibitors and antioxidants at the concentrations used were not toxic to the cells (data not shown).

Cell culture

Human umbilical vein endothelial cells (HUVEC), human glioma M059K cells, Chinese hamster ovary (CHO) cells deficient in endogenous β_3 and human prostate cancer epithelial cell lines LNCaP and PC3 were cultured as previously described (Hatziapostolou et al., 2005; Koutsioumpa et al., 2013; Tsirmoula et al., 2012). Stable CHO clones expressing wild-type β_3 or mutant β_3Y773F/Y785F were generated as previously described (Mikelis et al., 2009). Cell culture reagents were from BiochromKG (Berlin, Germany). All cultures were maintained at 37 °C, 5% CO_2 and 100% humidity. When cells reached 80–90% confluence, they were serum-starved for 16 h before performing any further experiments.

Migration assay

Migration assays were performed as previously described (Koutsioumpa et al., 2013) in 24-well microchemotaxis chambers (Corning, Inc., Lowell, MA, USA) using uncoated polycarbonate membranes with 8-μm pores. Serum-starved cells were harvested and resuspended at a concentration of 10^5 cells/0.1 ml in serum-free medium containing 0.25% bovine serum albumin (BSA). The bottom chamber was filled with 0.6 ml of serum-free medium containing 0.25% BSA and the tested substances. The upper chamber was loaded with 0.1 ml of medium containing the cells and incubated for 4 h at 37 °C. After completion of the incubation, the filters were fixed and stained with 0.33% toluidine blue solution. The cells that migrated through the filter were quantified by counting the entire area of each filter, using a grid and an Optech microscope (Optech Microscope Services Ltd., Thame, UK) at an ×20 objective.

RNA interference

HUVEC were grown to 50% confluence in medium without antibiotics. Transfection was performed in serum-free medium for 4 h using annealed RNA for RPTPβ/ζ at the concentration of 50 nM and jetSI-ENDO (Polyplus Transfection, Illkirch, France) as transfection reagent (Polykratis et al., 2005), or XO at the concentration of 50 nM and Lipofectamine® RNAiMAX transfection reagent (Life Technologies). Cells were incubated for another 48 h for RPTPβ/ζ and 72 h for XO in serum-containing medium and serum starved before further experiments. Double-stranded negative control siRNA was used in all experiments.

Assay of intracellular ROS production

ROS were assayed using the ROS-sensitive fluorescent dye carboxy-H_2DCFDA, as previously described (Hatziapostolou et al., 2006). Cells were seeded in 12-well plates at a concentration of 2×10^5 cells/well. Twenty four hours after seeding, LNCaP and PC3 cells were directly assayed for carboxy-DCF fluorescence, as described below, while HUVEC, M059K and CHO cells were serum-starved and then incubated in serum-free medium containing 0.25% BSA and PTN for the indicated periods of time. When used, antioxidants were added into the cell culture medium 30 min prior to PTN stimulation. At the end of the incubation period, cells were washed with phosphate-buffered saline (PBS) pH 7.4, and then incubated in the dark for 15 min in Ham's F-12 lacking phenol red, containing 50 μM carboxy-H_2DCFDA. Cells were released with trypsin/EDTA and lysed. Soluble extracts were prepared by centrifugation for removal of cell debris and fluorescence intensity was determined spectrophotometrically, using an excitation wavelength of 485 nm and emission wave-length of 500 nm. Cell lysates were analyzed for protein content using the Bradford method, and carboxy-DCF fluorescence was normalized for total protein content.

Statistical analysis

The significance of variability between the results of each group and its corresponding control was determined by unpaired t test. Each experiment included triplicate measurements for each condition tested, unless otherwise indicated. All results are expressed as mean ± s.e.m. from at least three independent experiments.

Results

PTN increases carboxy-DCF fluorescence in a concentration- and time-dependent manner

Treatment of HUVEC with PTN at the concentration of 100 ng/ml that causes maximal stimulation of HUVEC migration (Polykratis et al., 2005) resulted in a time-dependent increase in carboxy-DCF fluorescence, which was maximal 10 min after PTN stimulation (Fig. 1A). Treatment of cells with different concentrations of PTN for 15 min resulted in a concentration-dependent increase in carboxy-DCF fluorescence (Fig. 1B).

Similarly to HUVEC, PTN increased carboxy-DCF fluorescence in a concentration- and time-dependent manner also in human prostate cancer LNCaP cells (Supplementary Figure S1). We have previously shown that down-regulation of endogenous PTN in human prostate cancer LNCaP (Hatziapostolou et al., 2005) or PC3 (Tsirmoula et al., 2012) cells results in decreased ability of the cells to migrate and form colonies in soft agar in vitro, increased apoptosis and decreased growth in immunocompromised rats in vivo. In the present study, we used the same cells stably expressing antisense PTN and investigated whether endogenous PTN levels correlated with intracellular carboxy-DCF fluorescence. As shown in Fig. 2, intracellular carboxy-DCF fluorescence was decreased in both LNCaP and PC3 cells with decreased expression of endogenous PTN. In cells transfected with the appropriate control vector, carboxy-DCF fluorescence was similar to those in the corresponding non-transfected cells.

RPTPβ/ζ is involved in PTN-induced elevation of carboxy-DCF fluorescence

Since RPTPβ/ζ is one of the main PTN receptors, we studied whether RPTPβ/ζ is involved in PTN-induced carboxy-DCF fluorescence elevation. Treatment of HUVEC with the protein tyrosine phosphatase

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