the toxic change in microglia or act only on the toxic subtypes of microglia.

Future prospects of studies of postmortem PD brains

Biochemical and molecular biological studies of postmortem brains in PD have greatly contributed to our understanding of PD pathogenesis at the molecular level, though interpretation of the data must be made with caution due to the complexity of factors in the postmortem human brain.

First, establishment of brain banks including control brains are the most important to obtain an adequate number of samples. It is desired to establish the same and common system of brain banks in order to exchange brain samples among many brain banks.

Second, precise clinical records of the patients including drug administration are essential.

Third, one must consider that punched-out brain tissues, however small the sample is, contain various neurons and glial cells. Therefore, the precise brain location for punching-out tissues becomes highly critical. Various micropunching techniques have been developed, owing to the increased sensitivity of analytical systems, e.g., high-performance liquid chromatography (HPLC) with micro-bore columns (internal diameter <1–2 mm) for various neurotransmitters, and RT-PCR for the assay of mRNA contents. For example, the detection limit for catecholamines by using micro-bore HPLC is ~50 fmol (Nagatsu and Kokjima, 1988). Thus, even tissues of ~mg order can be analyzed.

Kanazawa's group for the first time performed single-cell analysis of CAG repeats in brains of two patients with dentatorubral-pallidoluysian atrophy (DRPLA) by using a newly developed excimer laser microdissection system to analyze somatic mosaicism in their brains (Hashida et al., 2001). They also provided the first quantitative measurements of the mRNA expression profile of AMPA receptor subunits in human single neurons from patients with amyotrophic lateral sclerosis (ALS) by means of quantitative RT-PCR with a laser micro-dissector (Kawahara et al., 2003).

Sawada's group has also established a method for single-cell analysis by laser capture micro-dissection for identification of cells by immunohistochemistry. Analysis of the effects of various biologically active compounds can now be carried out on a single cell or the same group of cells, which are isolated by laser capture microdissection and identified by immunohistochemical staining. Biochemical studies on postmortem human brains at the cellular level will further contribute to elucidation of the molecular pathology of PD, AD, and other neurodegenerative and neuropsychiatric diseases.

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STIMULUS DEPENDENT REGULATION OF

THE PHAGOCYTE NADPH OXIDASE

BY A VAV1, Rac1, AND PAK1 SIGNALING AXIS

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Running Title: PAK1 activates p47phox and NADPH oxidase

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The p21-activated kinase-1 (PAK1)[1] is best known for its role in regulation of cytoskeletal and transcriptional signaling pathways. We show here in the microglia cell line Ra2 that PAK1 regulates NADPH

Abbreviations; AA, Arachidonic acid; BIM-1, Bisindolylmaleimide-1; cyt b_{558}, flavocytochrome b_{558}; GEF, GTP exchange factor; LIMK, LIM kinase; Luminol E-CL, luminol-enhanced chemiluminescence; NOS, NO Synthetase; PAK, p21-activated kinase; PBD, p21-binding domain.

oxidase (NOX-2) activity in a stimulus-specific manner. Thus, conditional expression of PAK1 dominant-positive mutants enhanced, while dominant-negative mutants inhibited, NADPH oxidase-mediated superoxide generation following FMLP or PMA stimulation. Both Rac1 and the GTP exchange factor VAV1 were required upstream signaling proteins in the FMLP-induced activation of endogenous PAK1. In contrast, PAK1 mutants had no effect on superoxide

generation downstream of FcγR signaling during phagocytosis of IgG-immune complexes. We further present evidence that the effect of PAK1 on the respiratory burst is mediated through phosphorylation of p47phox, and show that expression of a p47phox (S303/304/320D) mutant, which mimics phosphorylation by PAK1, induced basal superoxide generation *in vivo*. In contrast PAK1 substrates LIMK1 or RhoGDI are neither likely to contribute to the PAK1 effect on NADPH oxidase activation. Collectively, our findings define a VAV1-Rac1-PAK1 signaling axis in mononuclear phagocytes regulating superoxide production a stimulus-dependent manner.

Introduction

The superoxide-generating phagocyte NADPH oxidase (NOX-2) consists of integral membrane subunits gp91phox and p22phox, and cytosolic subunits p40phox, p47phox, p67phox, and the small GTPase Rac(1 or 2). The gp91phox and p22phox proteins are permanently associated with each other in the membrane to form the catalytic flavocytochrome b_{558} core (cyt b_{558}) through which electrons from NADPH are transported to reduce molecular oxygen to superoxide on the external side. Only in activated phagocytes do the cytosolic factors translocate to the membrane to interact with cyt b_{558} and initiate superoxide production (39). *In vitro*, purified membranes containing cyt b_{558} demonstrate superoxide production when reconstituted with high

concentrations of p67phox and prenylated, GTP-loaded Rac1 (38), as these two cytosolic factors regulate electron transfer from NADPH to cyt b_{558} (51). But *in vivo*, p47phox is essential for NADPH oxidase activity, and serves to guide the p67phox subunit to the membrane. Upon cell activation, p47phox undergoes phosphorylation of multiple serine residues. This induces exposure of binding domains that can interact with p22phox (37) and phosphoinositide lipid products (53), thereby causing translocation of p47phox/p67phox to the membrane (14), presumably assisted by the actin cytoskeleton. Several kinases have been implicated in phosphorylation of p47phox including Akt (41,52), PKC isoforms (2), p38MAPK, and ERK1/2 (23).

The small GTPase Rac1, a required catalytic subunit of the NADPH oxidase holoenzyme, translocates to the membrane with identical kinetics as p47phox and p67phox (32), but seemingly independently thereof (27,30,43). Several guanine nucleotide exchange factors (GEF's) for Rac1 have been implicated in NADPH oxidase activity in neutrophils or *in vitro* including VAV1 (40) and P-Rex (7). An important effector molecule for Rac1 is the serine/threonine kinase p21-activated kinase (PAK1), which to a large extent mediates the effects of Rac1 and Cdc42 on the actin cytoskeleton (1). In the N-terminal part, PAK1 contains a small GTPase-binding site, the p21-binding domain (PBD; aa 67-150), partially overlapping with the so-called auto-inhibitory domain (AID; aa 83-149), which

is thought to engage in intramolecular bonding with the C-terminal kinase domain of PAK1 thereby preventing activity. However, binding of activated (GTP loaded) Rac1 or Cdc42 to the PBD relieves this allosteric inhibitory mechanism, freeing the kinase domain for activity (17).

It was recently shown that inhibition of PAK1 by the AID fragment exerts an inhibitory effect on the neutrophil respiratory burst (4). There are several putative mechanisms whereby PAK1 can affect NADPH oxidase activity, including RhoGDI phosphorylation (47) or regulation of actin dynamics involved in NADPH oxidase assembly. Furthermore, *in vitro* kinase assays have established that p47phox is a substrate of PAK1 (4,54), but how PAK1 modulates the respiratory burst *in vivo* (4), and whether such an effect is exerted through phosphorylation of p47phox, has remained unresolved.

In the following we have investigated the role of PAK1, and its immediate upstream regulators VAV1 and Rac1, in NADPH oxidase activation in murine microglia. Using lentivectors to achieve efficient conditional expression of wild type or dominant-negative or -positive mutants of PAK1, VAV1, and Rac1, we demonstrate that PAK1 function is required for NADPH oxidase-mediated superoxide production following FMLP stimulation, and that this PAK1 activity depends on VAV1 and Rac1 as upstream signaling proteins. In contrast, we found no evidence for a role of PAK1 in

IgG-immune complex-induced NADPH oxidase activation.

Materials and methods

The murine microglia cell line Ra2 (licensed by the Japan Science and Technology Agency, Patent ID US6.673,6,5; JP3410738; EP10/602,234) was maintained in MEM with 10% FCS, 1 ng/ml GM-CSF (Peprotech, UK), and 5 µg/ml bovine insulin (10,31). FMLP, PMA, luminol, HRP-II, insulin, arachidonic acid, and diphenyliodonium (DPI) were purchased from Sigma-Aldrich. Bisindolylmaleimide-1 (BIM-1) and L-NMMA were from Calbiochem. Rabbit anti-PAK1 antibody, goat anti-phosphoT423 PAK1 antibody, and mouse anti-myc mAb (clone 9E10) were from Santa Cruz Biotechnology, rabbit anti-PAK1 antibody from Signal Transduction Laboratories, rabbit anti-PAK1 mAb from Abcam, rabbit anti-PAK1,-2,-3 from Chemicon, mouse anti-HA tag mAb from Sigma-Aldrich, and polyclonal rabbit anti-LIMK, and anti-phosphothreonine 508 LIMK antibodies were from Cell Signaling. Alexa 488-conjugated phalloidin, FcOxyburst, secondary Alexa 568 or 633-conjugated goat-anti-mouse, or goat-anti-rabbit antibodies for immunofluorescence and FACS were from Molecular Probes.

Lentivector construction

The cDNA of human origin for myc-tagged PAK1 wild type, the dominant inhibitory mutants PAK1-K299A, PBD, PBD-H83L, and the dominant positive PAK1-T423E and -H83,86L mutants (44), dominant negative myc-tagged VAV1-L213A (kindly provided by Dr. Amnon Altman, La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA), Rac1-V12 or Rac1-V12/Y40C (a generous gift of Dr. Mary Dinauer, Dept. of Microbiology and Immunology, Indiana University Medical School, I, USA), wild type or mutant p47phox(S303/304/320D) of human origin (a kind gift of Dr. Hideki Sumimoto, Medical Institute of Bioregulation, Kyushu University, Japan), or HA-tagged dominant negative Rac1-N17 (kindly provided by Dr. Emanuelle Caron, Division of Cell and Molecular Biology, Imperial College, London, UK) were PCR amplified with primers to generate 5' and 3' restriction enzyme sites compatible with BamHI/SalI cloning into the lentiviral vector pLOX TW under the control of a tetracycline-responsive promotor (5). Human p47phox-GFP cDNA (a kind gift of Dr. Lance Terada, University of Texas, Southwestern Medical Center, Dallas, TX, USA) was PCR amplified with primers for directional TOPO cloning into the lentiviral vector pLenti-6/V5/TOPO (Invitrogen). All constructs were verified by sequencing.

Lentivirus production and cell transduction

Calcium phosphate transfection of HEK 293T producer cells and lentivector production has been described elsewhere (31). A Ra2 cell line referred to as 045 was established by transduction with lentivector pLOX TW rtTA (5), which expresses the tetracycline-inducible transactivator protein (rtTA). Following transduction of Ra2 045 with GFP-expressing vector pLOX TW

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GFP, more than 95% of the cells expressed high levels of GFP after overnight induction with 0.1-0.3 µg/ml doxycyline, conditions which were also used for induction of all rtTA-regulated transgenes in this study. By extrapolating results obtained from titering of pLOX TW GFP virus on Ra2 045 cells, we estimate that cells have received pLOX TW-PAK1, VAV1, Rac1, and p47phox lentivectors in a concentration of 20-40 MOI, which resulted in high level expression of transgene in more than 90% of cells as estimated by immunofluorescence and FACS analysis with anti-myc or anti-HA antibodies. In case of double transduction, PAK1-expressing Ra2 cells were superinfected with VAV1-L213A, Rac1-N17 or p47phox-GFP-expressing lentivectors as specified.

Cell lysis, immunoprecipitation, and 2D gel electrophoresis

To analyze phosphorylation of endogenous murine PAK1, Ra2 microglia in suspension in HBSS were stimulated with 4 µM FMLP at 37°C, and aliquots withdrawn at intervals plunged into ice-cold PBS to stop the reaction. Following centrifugation, and lysis of pellet in RIPA buffer (NaCl 150 mM, 1 mM EDTA, 0.1% SDS, 1 % deoxycholate, 1% NP-40, MOPS 50 mM, pH 8) with phosphatase and protease inhibitors (Sigma-Aldrich), immunoprecipitation with anti-PAK1 antibodies was carried out as described below. To analyze the effect of dominant-negative VAV1 and Rac1 on PAK1 activation, Ra2 microglia over-

expressing PAK1 wild type alone, or in combination with either dominant negative VAV1-L213A or Rac1-N17, were suspended in HBSS and stimulated with 2.5-5 µM FMLP at 37°C for 2 min. Stimulation was stopped by addition of a large volume of ice-cold PBS. Following centrifugation and lysis of cell pellet in RIPA buffer, the precleared lysate was incubated with protein A-conjugated sepharose beads and rabbit anti-PAK1 antibodies (4 µg/ml) overnight at 4°C. Beads were washed in RIPA buffer, and resuspended in Laemmlie sample buffer for subsequent electrophoresis, and western blotting with rabbit or goat antibody specifically recognizing PAK1 phosphorylated at T423, or mouse antibody recognizing the myc-tag of PAK1 wild type. For 2D-gel electrophoresis, Ra2 microglia were harvested and washed in PBS, pelleted, and lysed in 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3-10, and 40 mM Trizma base, pH 10.1. After lysis, reduction with 4 mM tributylphosphine, and alkylation of the samples with 15 mM iodoacetamide, aliquots were adjusted for protein concentration (Bio-Rad) and appropriate ampholytes (Bio-Rad) added. Then, 11 cm pH 3-10 IPG strips (Bio-Rad) or pH 6-11 IPG-strips (Sigma Aldrich) were equilibrated overnight with 150 µl of lysate containing 300 µg of protein. Alternatively, for p47phox-GFP immunoprecipitates 11 cm, pH 5-8 or pH 4-7 IPG strips (Bio-Rad) were used. The next day isoelectric focusing (15-20.000 Vhrs for neutral pH strips and

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70-80.000 Vhrs for pH 6-11 strips), and second dimension electrophoresis on 12.5% SDS-PAGE polyacrylamide gels was performed. Protein was subsequently semidry-transferred to PVDF membranes, and western blotted with anti-RhoGDI mAb (Transduction Laboratories) or polyclonal rabbit anti-p47phox antibodies (Santa Cruz; and kindly provided by Dr. Bill Nauseef, University of Iowa, Iowa City, IA, USA, and Dr. Frans Wientjes, Dept. of Medicine, University College London, UK). Signal was developed with appropriate secondary HRP-conjugated antibodies using ECL (Amersham).

Measurement of superoxide production

Luminol-enhanced chemiluminescence (Luminol E-CL) was used to measure FMLP or PMA-induced superoxide release as previously described (8) with the exception that luminescent signal was measured in a thermo stated Synergy HT microplate reader equipped with an injection module. Ra2 microglia in HBSS buffer on ice were warmed 1-2 minutes in a 37°C water bath before dispension into microtiter plates (Wallac black isoplates) at 100,000 cells/well. Subsequently, superoxide production of cells in suspension (plates were intermittently shaken to prevent cell lodging) was measured every 5-9 seconds at 37°C before and after stimulation with 4 µM FMLP or 100 ng/ml PMA (final concentrations) delivered through the injector module. Alternatively, Ra2 cells were preincubated with or without 200 nM

BIM-1 for 5 minutes before injection of arachidonic acid at a final concentration of 10 µM and measurement as above. To measure superoxide emitted during FcγR-mediated phagocytosis we used 5,7,-DHCF-conjugated BSA-IgG immune complexes (FcOxyburst, Molecular Probes), which form aggregates sufficiently large to be phagocytosed. One million Ra2 cells were incubated with FcOxyburst at a concentration of 50 µg/ml in a volume of 300 µl HBSS on ice for one hour, briefly centrifuged at low speed, resuspended, and then kept on ice in the dark until FACS analysis. Reaction was started by adding an excess of 37°C HBSS containing a low concentration of goat-anti-rabbit Alexa 633 antibodies to identify and gate cells with bound IgG aggregates. The cells were placed in a 37°C thermo stated chamber of a Beckton-Dickinson FACS Aria equipped with laser lines of 488 and 633 nm to excite 5,7,-DHCF and Alexa 633, respectively, and emission filters for FITC and APC detection, respectively. Cells with bound IgG aggregates (ca. 50% of total population, little variation between cell lines) were gated, and fluorescence intensity of 5,7,-DHCF was sampled for 3-5000 cells every third minute for up to 9 minutes. Mean fluorescence intensity of goat-anti-rabbit Alexa 633 after 30-60 seconds of mixing with antibodies was taken as measure of IgG-aggregate binding capacity of the cells, and 5,7,-DHCF fluorescence normalized to this value. Under the conditions used, 10 µM DPI or 1 mM L-

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NMMA inhibited 5,7,-DHCF fluorescence emission by 90 and 52%, respectively.

Immunofluorescence

Ra2 cells were fixed in 2% paraformaldehyde in phosphate buffer, pH 7.2, permeabilized with 0.2% saponin, and immunocytochemistry essentially performed as described (48) with mouse anti-myc mAb to detect PAK1, mouse anti-HA mAb to detect Rac1-N17, and Alexa 488-conjugated phalloidin to visualize fibrillary actin. Images were acquired with a Zeiss LSM510 confocal laser scanning microscope with a C-Apochromat X63, 1.2 oil immersion objective, using the 488 nm argon and 543 nm helium–neon laser lines for excitation of Alexa 488 and 568, respectively. Confocal sections (1.0-1.5 µm) were collected and saved as 512x512-pixel images at 8-bit resolution before import into Adobe Photoshop for compilation.

Results

Conditional expression of wild type and mutant PAK1 protein in murine microglia cell line Ra2

The established Ra2 murine microglia cell line (10) has been extensively used in our laboratory as it has a morphological phenotype resembling that of primary microglia, and has retained capacity for stimulated cytokine (10) and superoxide production (31). We investigated PAK isoform expression in these cells using PAK1-specific antibodies and a pan-PAK antibody recognizing a domain highly conserved in PAK-1, -2, and -3 isoforms. Human HL60 cells were included for comparison. As seen in Figure 1A, Ra2 cells expressed only PAK1 protein while HL60 cells expressed PAK1, and either PAK-2 or -3. For further analysis Ra2 microglia were transduced with lentivector carrying the tet-transactivating response protein (rtTA) under a CMV promotor and the resulting subline named Ra2 045. These cells were subsequently superinfected with lentiviral vectors expressing human wild type or mutant PAK1 protein including PAK1-K299A, PBD (p21-binding domain), PAK1-T423E, and PAK1-H83,86L (see Table I for an overview of mutants used in this study) under the control of the rtTA/doxycycline-responsive Tet promotor (5). N-terminal myc-tagging allowed for easy detection of transgene expression by Western blotting and immunofluorescence (see Figure 1B and C, and Figure 2).

Morphological effect of PAK1 wild type and mutant protein in microglia

PAK1 is well known to regulate cytoskeletal dynamics in mesenchymal cell types (21,22,26). To further verify effect of expressed PAK1 transgenes we therefore examined F-actin distribution by phalloidin staining in Ra2 cells expressing the various PAK1 mutants. Neither PAK1 wild type, nor the K299A, or PBD-H83L dominant negative mutant proteins, had any major effect on overall structure of the actin cytoskeleton (Figure 2), but could be seen to colocalize with actin in cortical structures. In contrast, PAK1-T423E had a tendency to induce plasma membrane "blebbing", while the other active mutant PAK1-H83,86L greatly increased the extension of broad lammellipodia. The blebbing induced by PAK1-T423E expression was only present in a subpopulation of cells and did not result in increased cell death. Interestingly, this phenotype is indistinguishable from that of overexpression of the PAK1 substrate LIMK1 alone (data not shown) and indicates that PAK1-T423E and -H83,86L differentially activate PAK1 targets that control cytoskeleton dynamics. For comparison, expression of dominant negative Rac1-N17 induced collapse of the actin cytoskeleton into a cortical patch causing rounding of cells and inhibition of lamellipodia, filopodia, and membrane ruffle formation. Collectively the data indicate that PAK1 mutants were functional in Ra2 cells, and resulted in specific cytoskeletal

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phenotypes. As there is a correlation between actin dynamics and NADPH oxidase function (11,18,42) all subsequent analysis on the function of NADPH oxidase in the Ra2 cells was performed with the cells in suspension to minimize the interference from these different PAK1-mediated morphological phenotypes.

PAK1 mutants alter FMLP-mediated NADPH oxidase activation

We have previously shown that Ra2 microglia express all subunits of the phagocyte NADPH oxidase and produce superoxide in response to common stimulators of the respiratory burst (31) including FMLP, which in neutrophils is known to induce PAK1 activation (54). We therefore examined the FMLP-induced respiratory burst by luminol-enhanced chemiluminescence (luminol E-CL) in Ra2 cells transduced with wild type or mutant PAK1, including the auto-inhibitory PBD fragment of PAK1. This region of PAK1 (aa 67-150) contains both the auto-inhibitory domain of PAK1 (also called AID), and binding sites for small GTPases Rac1 and Cdc42. As shown in Figure 3A and B, expression of PAK1 wild type and the K299A mutant did not have a major effect on the respiratory burst, although PAK1-K299A consistently delayed the response. In contrast, expression of the PBD fragment inhibited superoxide production almost completely, and expression of PAK1-T423E and -H83,86L caused more than a two-fold increase in superoxide release. To exclude

that PBD, which binds both Rac1 and PAK1, was exerting its inhibitory effect by sequestering GTP-loaded Rac1, we also analyzed the effect of the PBD-H83L mutant which does not interact with Rac1, but retains binding and inhibitory activity towards PAK1. As seen in Figure 3C, expression of PBD-H83L caused a dose-dependent decrease in superoxide generation, showing that the negative effect of PBD is mediated by repression of PAK1 function. In this figure and the following we have used luminol E-CL as a dynamic and highly sensitive probe for superoxide production. Depending on the presence of intracellular peroxidases and NO synthetase, luminol may also react with hydrogen peroxide, hydroxyl radicals, and peroxynitrite, albeit with different quantum yields. We have previously observed that that addition of L-NMMA, an inhibitor of NO synthetase, reduces the luminol E-CL signal with 20-30% (31). As the signal is blocked 90-95% by superoxide dismutase, DPI, or expression of Rac1-N17, superoxide is clearly critical for luminol E-CL in Ra2 cells, and we assume the L-NMMA sensitive fraction of the E-CL signal represents formation of peroxynitrite, the reaction product of superoxide and NO. As shown in Supplementary Figure 1, the mean L-NMMA/control ratio of the E-CL signal did not significantly differ between control cells and the different PAK1 cell lines. This indicates that the luminol E-CL assay in Ra2 cells measures preferentially superoxide, and that differences in luminol E-CL signal

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observed reflects a differential effect of PAK1 mutants on NADPH oxidase activity, and only to a minor extent NO production.

Rac1 GTP-exchange factor VAV1 is required for FMLP-induced PAK1 activation and NADPH oxidase activation in Ra2 microglia cells

Several GEF's have been linked to NADPH oxidase activation in neutrophils, including VAV1 (40). To investigate whether the same GEF would be responsible for activation of Rac1 in its role as a catalytic subunit of NADPH oxidase, as well as an activator of PAK1, Ra2 cell lines conditionally expressing dominant negative Rac1-N17 or dominant negative VAV1-L213A (Figure 4A) were analyzed for superoxide production and PAK activation following FMLP challenge. As seen in Figure 4B, VAV1-L213A exerted a dose-dependent negative effect on the magnitude of superoxide release, attaining almost complete inhibition at the highest expression levels. This indicates that VAV1 in microglia is an important GEF involved in NADPH oxidase activation following stimulation with FMLP.

When Ra2 microglia were stimulated with FMLP, endogenous PAK1 was transiently activated (Figure 5A, and B) as determined by antibodies specific to phosphothreonine-423, which is autophosphorylated upon PAK1 activation (17). To decide whether the FMLP-induced PAK1 activation observed was a result of VAV1 and Rac1 signaling we created cell

lines co-expressing PAK1 wild type protein with either Rac1-N17 or VAV1-L213A (all conditionally expressed). As seen in Figure 5C and D, stimulation with FMLP induced auto-phosphorylation of PAK1 in cells expressing PAK1 wild type alone. However, co-expression of either dominant negative VAV1 or Rac1 abrogated the FMLP-induced PAK1 auto-phosphorylation, indicating that VAV1 and Rac1 are necessary also for FMLP-induced PAK1 activation.

Effect of PAK1 wild type or mutants on PMA-stimulated NADPH oxidase activation

Phorbol esters cause phosphorylation of p47phox and evoke a solid respiratory burst in phagocytes. When Ra2 microglia in suspension were stimulated with PMA (Figure 6) the positive effect of the PAK1-H83,86L mutant on the magnitude of superoxide production was again evident, and additionally the lag time from stimulus to commencement of superoxide production was decreased. Curiously, the dominant negative K299A mutant, which did not significantly depress the FMLP-mediated superoxide release, caused a 50% decrease in the case of PMA stimulation. Further, the strong enhancing effect of PAK1-T423E seen after FMLP stimulation was absent and PBD-H83L, which exerted a strong inhibition of the FMLP response, caused only a slight inhibition of the PMA-induced response. As before, Rac1-N17 entirely blocked superoxide generation. It can be

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added that in a FACS-based assay for superoxide production we found no effect of PAK1 mutant expression following stimulation of NADPH oxidase by IgG-immune complexes, which bind and signal through FcγR's (SUPPL. MATERIAL, Figure 2).

Effect of dominant positive Rac1-V12 and Rac1-V12/Y40C mutants on NADPH oxidase activation.

To further substantiate a role for PAK1 in NADPH oxidase activation we created Ra2 cell lines conditionally expressing Rac1-V12 or Rac1-V12/Y40C. Both of these Rac1 mutants are constitutively active, but the Rac1-V12/Y40C mutant is incapable of binding and thereby activating endogenous PAK1. As seen in Figure 7A,B, both Rac1 mutants inhibited the FMLP-induced respiratory burst with ca. 50%. When PMA was used as stimulus, however, both of the mutants increased the superoxide release, Rac1-V12 being the most effective (Figure 7C).

Expression of dominant positive PAK1 mutants increase phosphorylation of p47phox, but not RhoGDI

A direct way for PAK1 to influence the respiratory burst is by phosphorylation and activation of p47phox, which is a substrate of PAK1 *in vitro* (54).

We therefore examined phosphorylation of endogenous p47phox in Ra2 cells expressing PAK protein by 2D-gel electrophoresis and western blotting. Cell lysates were subjected

to isoelectric focusing using pH 3-10 or pH 6-11 strips as described in materials and methods, and subsequently electrophoresed by SDS-PAGE and transferred to PVDF membranes for western blotting with polyclonal anti-p47phox antibodies. As seen in Figure 8A, only PAK-H8386L expressing cells showed consistent evidence of phosphorylation of p47phox, as judged by the appearance of two-three anodic (more acidic) species of p47phox not present in Ra2 045 control cells, or PAK1-K299A or PAK1 WT-expressing cells. These data are supported by initial studies of p47phox phosphorylation in transfected HEK293 cells and Ra2 cells co-expressing the different PAK proteins with a GFP-p47phox fusion protein (SUPPL. MATERIAL, Figure 3 and 4). In contrast, by 2D-gel electrophoresis we found no indication that any of the PAK1 mutants appreciably affected phosphorylation of endogenous RhoGDI (Figure 8B), which constitutes another PAK1 target with potential to influence NADPH oxidase activation via regulation of Rac1 nucleotide exchange

PAK1-H83,86L expression synergizes with arachidonic acid in NADPH oxidase activation

It is largely accepted that phosphorylation causes p47phox to unfold from its closed conformation and expose binding sites for p22phox and phosphoinositides in the membrane (14,53). It has been proposed that p47phox unfolds in two successive steps (interruption of C- and N-terminal

interactions, respectively, with tandem SH3 domains in the middle of p47phox responsible for p22phox binding), based on the observation that low concentrations of arachidonic acid (AA), not sufficient for full p47phox unfolding, synergize with phosphorylation (by PKC) in NADPH oxidase activation (19). We therefore tested whether AA at low concentrations would synergize with dominant positive PAK1 mutants in the induction of NADPH oxidase activity. Because AA effects with regard to NADPH oxidase activation may in part be mediated through PKC activation (28) the experiments were performed in the presence of 200 nM BIM-1, which inhibits PKC isoforms with a $K_i \sim 10$ nm. As seen in Figure 9A, 10 µM AA did not cause a marked respiratory burst in PAK1 wild type or PAK1-T423E Ra2 cells, however, in PAK1-H8386L expressing cells AA caused a sharp immediate peak of superoxide production followed by a longer period with sustained activity (Figure 9A and B). Reversely, the PAK1-K299A mutant decreased the magnitude of the AA-induced peak response with ca. 50%. Next, we examined membrane association of a p47phox-GFP fusion protein in the different Ra2 PAK protein expressing cells by immunofluorescense. While association of p47phox-GFP with cellular membranes following treatment with 10 µM AA was enhanced over non-treated control cells (data not shown), and p47phox-GFP co-localized with a fraction of PAK1 at the membrane

(Figure 9, C), by visual inspection p47phox-GFP recruitment to the membrane and co-localization with PAK1 seemed independent of the PAK1 mutant expressed (data not shown). The p47phox residues phosphorylated by PAK1 in vitro have been mapped to serines 303, 304, 320 and 328 (4). We therefore expressed a p47phox mutant with serine to glutamate substitutions on residues 303, 304, and 328 (14) to mimic phosphorylation, and then tested superoxide release as before with luminol-E-CL. Figure 9, D and E show that both p47phox wild type and p47phox-S303/304/328D dramatically increased the respiratory burst of PMA or FMLP-stimulated Ra2 cells (ca. tenfold more than overexpression of gp91phox). The wild type p47phox protein most efficiently supported the respiratory burst, but notably the p47phox-S303/304/328D caused a basal release of superoxide not normally seen in unstimulated Ra2 microglia (Figure 9, D and E, and inset in 9D).

Discussion

We here show that PAK1 regulates FMLP and PMA-induced phagocyte NADPH oxidase (NOX-2) superoxide production. We decided to undertake an investigation of the *in vivo* role of PAK1 in NADPH oxidase regulation because i) PAK1 is known to phosphorylate p47phox *in vitro* (54), ii) PAK1 is Rac1-regulated, thereby constituting a potential link between the Rac and phox activation arms of NADPH oxidase assembly, and finally iii) in contrast to other known kinases of p47phox (see below), PAK1 is also a major regulator of the actin cytoskeleton, which directly or indirectly supports NADPH oxidase assembly and function (11,18,42). While this work was under way it was reported that introduction of the AID fragment of PAK1 (included in PBD used herein) into neutrophils inhibited the respiratory burst *in vivo* (4), and further, adenoviral delivery of PAK1-K299A in endothelial cells inhibited p47phox phosphorylation and superoxide production (29). We present here the most extensive evidence for a positive role of PAK1 in NADPH oxidase signaling via p47phox phosphorylation by showing that i) FMLP stimulation of microglia activates PAK1 through a VAV1 and Rac1-dependent pathway, and ii) active PAK1 mutants cause p47phox phosphorylation and preactivation, leading to iii) an increased respiratory burst, an effect iv) reproduced by a p47phox mutant that mimics PAK1 phosphorylation.

Involvement of PAK1 in NADPH oxidase activation is stimulus dependent

Constitutively active PAK1-T423E, and in particular PAK1-H83,86L, increased FMLP-induced NADPH oxidase activity, and conversely, PAK1-K299A and the PBD-H83L fragment negatively affected activity. PMA activates PKC, which is well known to participate in phosphorylation of p47phox (see (27) for references), and is generally considered to phosphorylate all serine residues required for activation of p47phox (20). Nevertheless, PAK1 also influenced PMA-induced NADPH oxidase activation, although the effects were less clear-cut than for the FMLP-stimulated response. Firstly, only PAK1-H83,86L, but not -T423E, enhanced the PMA-stimulated respiratory burst. Secondly, PAK1-K299A, which only retarded the FMLP-response, reduced the superoxide output following PMA stimulation with 50%, and finally, the strong inhibitory effect of PBD-H83L in the case of FMLP was absent for PMA. The paradoxical, inverted inhibitory effect of PAK1-K299A and PBD on FMLP and PMA-stimulated superoxide production, respectively (compare Figure 3B with 6B), can perhaps be explained by the recent uncovering of a binding interaction between p22phox and PAK1 (4). PAK is otherwise known to engage in direct binding with its molecular partners in multisubunit complexes, e.g. PAK1-COOL-Pix or PAK2-NEF-VAV1 (1). Therefore PAK1-K299A could exert its negative effect on PMA-induced NADPH oxidase activity, not on the

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level of (inhibition of) p47phox phosphorylation, but rather via a negative effect on the assembled oxidase by a steric hindrance mechanism, not exerted by the much smaller PBD fragment.

Although PAK1 is recruited to forming phagosomes (26,45,46), the association is transient (45), and at present the activation status and function of both PAK1 and VAV1 during FcγR-mediated phagocytosis of opsonized prey is unclear (12,49). We found no major effect of PAK1 mutant over-expression on the respiratory burst associated with phagocytosis of IgG-immune complexes, indicating that PAK1 is not required for NADPH oxidase activation downstream of FcγR signaling (Suppl. Figure, 2).

In PMA-stimulated Ra2 cells, peroxynitrite, the reaction product between superoxide and NO, contributes most significantly to the luminol E-CL signal immideately after stimulation (the oxidative burst) where the ratio between L-NMMA and control cell E-CL drops to ca. 0,5 before stabilizing around 0,75 (Suppl. Figure 1). Importantly, the ratio did not vary between control or the different Ra2 PAK1 cell lines. As the 'specific' iNOS inhibitor 1400W has no inhibitory effect on the luminol E-CL signal in Ra2 cells we assume the source of NO is either nNOS or eNOS, whose activity can be in part be regulated by serine and threonine phosphorylation (15,16,50). We cannot exclude that PAK mutants differentially activate NOS or cause its uncoupling (to

produce superoxide) but our control experiments show that NO production cannot account for the observed differences in the luminol E-CL signal. Further experimentation is needed to establish whether PAK1 directly or indirectly contributes to the phosphorylation state, and thereby activity, of NOS.

Can constitutively active Rac1 initiate NADPH oxidase activity in phagocytes?
Several GEF's have been proposed to mediate Rac nucleotide exchange in the setting of respiratory burst induction in neutrophils, including Tiam1, P-Rex, and VAV1 (7,40,55). We show here that dominant negative VAV1-L213A (and expectedly Rac1-N17) inhibited the FMLP-stimulated activation of PAK1 and dose-dependently decreased the microglia respiratory burst. Therefore VAV1-generated GTP-Rac1 regulates NADPH oxidase activity both by being an essential subunit (51), as well as by contributing to PAK1 activation. This is supported by our demonstration in HEK293T cells that expression of constitutively active Rac1-V12, but not Rac1-V12/Y40C, which has lost its ability to interact with PAK1 (and p67phox), is sufficient to induce phosphorylation of p47phox to a level similar to that of dominant positive PAK1 mutants alone (Suppl. Figure 3). Similarly, Rac1-mediated PAK1 activation could explain the observed spontaneous activation of NADPH oxidase in a heterologous COS cell system expressing dominant positive

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VAV1 or Rac1 (33). However, although Rac1-V12 expression in Ra2 microglia more efficiently increased the PMA-induced superoxide production than Rac1-V12/Y40C (Figure 7), we observed no spontaneous NADPH oxidase activity above control levels for any of the Rac1-V12 mutants, in contrast to heterologous COS cells (33), indicating additional regulatory elements in phagocyte cells. Both Rac1-V12 dominant positive mutants inhibited the FMLP-induced respiratory burst *in vivo*, probably because of a requirement for full GDP/GTP cycles as proposed previously for small GTPases Rac2 and Rap1A (3,34).

Phosphorylation of p47phox by PAK1

It is currently thought that the primary role of p47phox is to transport and tether p67phox to cyt b_{558}. Phosphorylation of serines 303, 304 and 328 of p47phox is a requisite step in the membrane translocation and attachment to cyt b_{558} of p47/67-phox (6,14,53) by virtue of phosphorylation-induced uncovering of latent binding sites for p22phox (13,37) and phosphoinositol lipids (35,53) in the membrane. Several kinases have been implicated in p47phox phosphorylation leading to activation or priming of the NADPH oxidase including PKC isoforms (9,25,36), Akt (41,52), and MAP kinases (23). While a role for PAK1 in p47phox mobilization has been suggested by its *in vitro* phosphorylation of p47phox (54), we show here by 2D-gel electrophoresis and western blotting that expression of PAK1-H83,86L, and to a lesser extent PAK1-

T423E, caused p47phox phosphorylation *in vivo*. Careful mapping has demonstrated that serines 303, 304, 320, and 328 (in particular the latter) of p47phox serve as phospho-acceptor sites for PAK1 *in vitro* (4). While full activity (eliciting superoxide production) of p47phox requires additional phosphorylation of S379 (24), the phosphorylation mediated by PAK1 should be sufficient to relieve intramolecular restraints in p47phox to allow for p22phox (14) and inositol lipid (53) binding. As shown here introduction of a p47phox(S303/304/328D) mutant as a p47phox phosphomimetic was sufficient to cause a marked superoxide production in Ra2 cells even under unstimulated conditions. Phosphorylation of p47phox by PAK1-H83,86L did not cause a consistent basal superoxide release, but did cause partial activation of p47phox since PAK1-H83,86L synergized with low concentrations of AA (which cause only partial unfolding and preactivation of p47phox (19,53)) in NADPH oxidase superoxide production. While we found no evidence for PAK1 phosphorylation of RhoGDI, which releases bound Rac1-GDP for subsequent interaction with GEF's (47), we cannot exclude that one or more of PAK1's many other substrates (e.g. cytoskeleton-related proteins such as cortactin or filamin (1)) may also influence NADPH oxidase assembly and activation. The important PAK1 substrate LIMK-1, which controls cofilin-dependent actin depolymerization does not contribute to the PAK1 effects observed in this study

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(manuscript in preparation). Therefore, for
the reasons laid out above we greatly favour
the idea that the positive effect of PAK1 on
NADPH oxidase activity is mainly mediated
by direct phosphorylation and preactivation
of p47phox.

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