

MEETING GREMI 2007

OXIDATIVE STRESS & INFLAMMATION

January 26, 2007, Pasteur Institute - Paris

Organizing Committee: S. Chollet-Martin, V. Witko-Sarsat, V. Lagente
X. Norel, M. Si-Tahar, M. Chignard

9h30-9h40 Introduction by Michel Chignard, President of GREMI

Session 1

Moderators: V. Lagente / W. Reynolds

9h40-10h10 **K-H. Krause:**
Radicals are everywhere: the NOX family of ROS-generating NADPH oxidases

10h10-10h25 Z. Taoufiq:
Thwarting Plasmodium falciparum-induced oxidative stress within endothelial cell:
Prospects in severe malaria therapy

10h25-10h40 C. Villiers:
Analysis of ROS production by immature and activated murine dendritic cells:
modification of NADPH-oxidase activity and localization upon Toll-Like Receptor
stimulation

10h40-11h20 *Coffee break - posters - exhibitors*

Session 2

Moderators: S. Chollet-Martin / K-H. Krauze

11h20-11h50 **J. El-Benna:**
**Regulation of neutrophil NADPH-oxidase activity by pro-inflammatory and
anti-inflammatory cytokines**

11h50-12h05 M.J. Stasia:
Leu505 of Nox2 is crucial for optimal p67phox-dependent activation of the
flavocytochrome b558 during phagocytic NADPH oxidase assembly

12h05-12h20 V. Ollivier:
Monocytes down-regulate platelet activation induced by a collagen surface

12h20-12h35 A.P. Gobert:
Inhibition of nitric oxide production in human intestinal epithelial cells by
enterohaemorrhagic Escherichia coli results in increased Shiga-toxin synthesis

12h35-14h30 *Lunch - posters - exhibitors*

Session 3

Moderators: F. Morel / V. Witko-Sarsat

14h30-15h00 W. Reynolds:

Myeloperoxidase as a mediator in inflammatory disease

5h00-15h15 A. Haegens:

Exposure of alveolar and bronchial epithelium to myeloperoxidase modulates epithelial cell responses to pro-inflammatory stimuli

15h15-15h30 N. Charni-Ben Tabassi:

Nitrosylated N-telopeptide of type III collagen (IINys): A new specific biochemical marker of oxidative-induced synovial tissue metabolism in arthritis

15h30-15h45 M. Costantino:

Sulphur mud-bath therapy in treatment of osteoarthritis: possible antioxidant role

15h45-16h25 Coffee break - posters - exhibitors

Session 4

Moderators: M. Chignard / M-A. Gougerot-Pocidalo

16h25-16h55 J. Boczkowski:

Heme oxygenase: from heme degradation to modulation of redox signaling and inflammation

16h55-17h10 M. Djavaheri-Mergny:

Regulation of macroautophagy by NF-kappaB transcription factor

17h10-17h25 F. Rannou:

Pharmacological induction of heme oxygenase-1 decreases the acute phase of inflammatory arthritis

17h25-17h40 S. Rémy:

Carbon monoxide generated by heme oxygenase-1 activity confers tolerogenic capacity to dendritic cells

17h40-17h55 P. Winyard:

Upregulation of nitric oxide in the knee-joints of rheumatoid arthritis patients: contribution to a neurogenic pathway of inflammation

17h55-18h00 Closing Remarks

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ORAL PRESENTATIONS

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SESSION 1

Radicals are everywhere: the NOX family of ROS-generating NADPH oxidases

Karl-Heinz Krause (Dept. of Pathology, Geneva University Hospitals)

Designed generation of reactive oxygen species (ROS) by the phagocyte NADPH oxidase was for a long time considered as an oddity restricted to professional phagocytes. Over the last years, 6 homologues of the cytochrome subunit of the phagocyte NADPH oxidase were found: NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2. Together with the phagocytes NADPH oxidase itself (NOX2/gp91 phox), these homologues are now referred to as the NOX family of NADPH oxidases. NOX enzymes share the capacity to transport electrons across the plasma membrane and to generate superoxide and other down-stream reactive oxygen species. But activation mechanisms and tissue distribution of the different members of the family are very different, suggesting distinct physiological functions. NOX family enzymes are likely to be involved in a variety of physiological events, including host defense, post-translational processing of proteins, and blood pressure regulations. Also, an increase and a decrease in the function of NOX enzymes can lead to disease processes.

Reference: Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Reviews*, 2007; 87: 245-313.

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Thwarting Plasmodium falciparum-induced oxidative stress within endothelial cell: Prospects in severe malaria therapy

Zacharie TAOUFIQ, Marc Conti, Maurel Tefit, Dominique Mazier, Ioannis Vouldoukis

INSERM U511, Faculté de Médecine Pitié-Salpêtrière, Université Pierre et Marie Curie-Paris 6 - 75013 Paris

Malaria remains a major threat to public health with 40% of the world population currently at risk. Each year 500 million clinical cases are reported and more than a million people die from severe complications associated with a Plasmodium falciparum infection. Acute clinical manifestations of malaria, such as cerebral malaria (CM) can appear unpredictably, leading to coma and death within hours if untreated. Despite administration of efficient anti-parasitic drugs in CM emergency treatments, 15-20% of lethality is still observed and neurocognitive sequelae reported. Additional therapeutic approaches are thus urgently needed to improve the outcome of the disease.

The pathogenesis of fatal cases of malaria relies essentially on the unique ability of P. falciparum parasitized red blood cells (pRBC) to adhere on microvascular endothelial cells via adhesion molecules, such as ICAM-1, VCAM-1 and E/P-Selectins. In this way parasites avoid spleen clearance by sequestration in post-capillary venules of various organs including the brain. However adhesion molecules have also been shown to have molecular signalling consequences. We have developed a human in vitro endothelial barrier/infected erythrocytes co-culture model for several years in our laboratory. We showed that pRBC contact specifically induces oxidative stress, pro-inflammatory genes expression, adhesion molecules upregulation (mainly ICAM-1) and caspases activation within endothelial cells. Consequent apoptosis was shown to involve the mitochondrial pathway through Bad and caspase 9. Adhesion of pRBC triggered mitochondrial depolarization and over-production of superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2).

Cytoplasmic Cu/Zn superoxide dismutase (SOD1) is now used as a treatment of numerous diseases including traumatic brain injury and ischemic stroke. We recently tested in our model the transient supplementation of SOD1 within endothelial cells and demonstrated that it strongly protected cells against P. falciparum induced oxidative stress. Our results clearly showed a strong anti apoptotic effect, likely through synergistic beneficial events to the endothelium with: 1) the decrease of $O_2^{\cdot -}$ concentration, 2) the production of nitric oxide (NO) derivatives, 3) the decrease of ICAM-1 expression and pRBC cytoadherence.

In conclusion, the protection of endothelial functions via antioxidant delivery may thwart parasite-induced microvascular stress and may constitute a relevant strategy in severe malaria treatment.

ABSTRACT 1

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Analysis of ROS production by immature and activated murine dendritic cells: modification of NADPH-oxidase activity and localization upon Toll-Like Receptor stimulation

Marie-José Stasia^{1,2}, Didier Grunwald³, Gaétan Gavazzi², Patrice N. Marche¹ and VILLIERS Christian¹

¹INSERM U548; CEA-G/DRDC/ICH,38054 Grenoble, France ; ²GREPI, MENRT EA2938 laboratoire d'Enzymologie, CHU, Grenoble, France ; ³INSERM EMI 0104 CEA-G/DRDC/TS, Grenoble, France

Dendritic cells (DCs) are known for their antigen-presenting capacity and their role in primary specific immune responses; DCs reside at the interface between innate and adaptive immunity. Immature DCs have the ability via pathogen recognition receptors, first to detect then to internalize and process foreign molecules for antigen presentation to T cells. NADPH oxidase is a crucial enzyme present in phagocytic cells, and accountable for the production of superoxide radicals (O₂⁻), the initial material for the generation of various reactive oxygen species including oxygen peroxide. Recently, we have shown that all the proteins included in the NADPH oxidase complex and usually found in phagocytic cells such as neutrophils, are immunodetected in murine DCs (Elsen et al. (2004). But very low extracellular O₂ production was measured on immature cells after PMA stimulation, even after a prolonged cellular activation in presence of LPS.

Here, we demonstrated that DCs are able to produce H₂O₂ after NADPH oxidase stimulation using various compounds: PMA, chemotactic peptides, PAF, or zymosan. Constitutive H₂O₂ production was also measured in DCs without stimulation. Both productions are oxidase-dependent as assessed by diphenylene iodonium (DPI) inhibition and by the absence of H₂O₂ production in gp91phox-KO murine DCs. In both cases, more than 50% of the produced H₂O₂ remained intracellular. Experiments using immature and LPS activated DCs loaded with dihydrorhodamine (DHR123), revealed that both DCs are H₂O₂-producing cells. Furthermore, using confocal microscopy, we have shown that the NADPH oxidase components of immature DCs were partly transferred from the cytoplasmic membrane to the membrane surrounding phagolysosomes after zymosan internalization. The activation of murine DCs by TLR agonists does not significantly affect the H₂O₂ production level. But, in LPS-activated DCs, some of the oxidase components are found in intracellular structures surrounding the nucleus. Our results support the hypothesis that the NADPH oxidase complex could likely play distinct roles in DCs depending on their maturation state, from bactericide activity in immature DCs to intracellular signaling in activated DCs. These two aspects of the NADPH oxidase activity of DCs are currently under investigation in our lab.

ABSTRACT 2

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SESSION 2

Regulation of neutrophil NADPH-oxidase activity by pro-inflammatory and anti-inflammatory cytokines

Jamel El-Benna (INSERM U773, Paris)

Neutrophils play a key role in host defenses against invading microorganisms and have a major role in inflammation. In response to a variety of agents, they release large quantities of superoxide anion (O_2^-) and other ROS in a phenomenon known as the respiratory burst. Neutrophil production of O_2^- is dependent on activation of NADPH oxidase, a multicomponent enzyme system that catalyzes NADPH-dependent reduction of oxygen to O_2^- . In resting cells NADPH oxidase is inactive and its components are distributed between the cytosol and membranes. When cells are activated, the cytosolic components (p47phox, p67phox, p40phox and Rac2) migrate to the membranes, where they associate with the membrane-bound component (flavocytochrome b558) to assemble the catalytically active oxidase. Upon NADPH oxidase activation, p47phox, p67phox, p40phox and p22phox become phosphorylated. P47phox phosphorylation on several serines plays a pivotal role in oxidase activation in intact cells. Neutrophil superoxide production can be potentiated by prior exposure to "priming" agents such as the pro-inflammatory cytokines GM-CSF, TNF and IL-8. The intimate mechanisms involved in the priming process are poorly understood. We and others have reported that priming of the human neutrophil respiratory burst by GM-CSF, LPS and TNF is associated with partial phosphorylation of the cytosolic NADPH oxidase component p47phox. Using peptide sequencing by tandem mass spectrometry we showed that GM-CSF and TNF induced phosphorylation of Ser345 on p47phox. As Ser345 is located in the MAPKinase consensus sequence, we tested the effects of MAPK inhibitors. Inhibitors of the ERK1/2 pathway abrogated GM-CSF-induced phosphorylation of Ser345, while p38MAPK inhibitor, abrogated TNF-induced phosphorylation of Ser345. This event was also inhibited in neutrophils by a cell-permeable peptide containing TAT-p47phox-ser345 sequence. ROS generation, phosphorylation of p47phox-Ser345, and phosphorylation of ERK1/2 and p38MAPK are increased in synovial neutrophils from rheumatoid arthritis (RA) patients. The TAT-Ser345 peptide inhibited ROS production by synovial neutrophils of these patients. The anti-inflammatory cytokine IL-10 inhibited GM-CSF-induced p47phox phosphorylation by inhibiting ERK1/2 activity. Convergent MAPK pathways on Ser345 are involved in GM-CSF- and TNF-induced priming of neutrophils and are activated in RA. Inhibition of the point of convergence of these pathways might serve as a novel anti-inflammatory strategy.

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LEU505 of NOX2 is crucial for optimal P67PHOX-dependent activation of the flavocytochrome B558 during phagocytic NADPH oxidase assembly

STASIA Marie-José

GREPI MENRT 2938, Centre CGD - Lab Enzymologie, BP217 - 38043 Grenoble Cedex 9

The role of Leu505 of Nox2 on the NADPH oxidase activation process was investigated. An X-CGD PLB-985 cell line expressing the Leu505Arg Nox2 mutant was obtained, exactly mimicking the phenotype of a previously published X91+CGD case. In a reconstituted cell-free system (CFS), NADPH oxidase and idonitrotetrazolium (INT) reductase activities were partially maintained concomitantly with a partial cytosolic factors translocation to the plasma membrane. This suggests that assembly and electron transfer from NADPH occurred partially in the Leu505Arg Nox2 mutant. Moreover, in a simplified CFS using purified mutant cytochrome b558 and recombinant p67phox, p47phox and Rac1 proteins, we found that the Km for NADPH and for NADH was about three times higher than those of purified WT cytochrome b558, indicating that the Leu505Arg mutation induces a slight decrease of the affinity for NADPH and NADH. In addition, oxidase activity can be extended by increasing the amount of p67phox in the simplified CFS assay. However, the maximal reconstituted oxidase activity using WT purified cytochrome b558 could not be reached using mutant cytochrome b558. In a three-dimensional model of the C-terminal tail of Nox2, Leu505 appears to have a strategic position just at the entry of the NADPH binding site and at the end of the alpha-helical loop (residues 484504), a potential cytosolic factor binding region. The Leu505Arg mutation seems to affect the oxidase complex activation process through alteration of cytosolic factors binding and more particularly the p67phox interaction with cytochrome b558, thus affecting NADPH access to its binding site.

ABSTRACT 3

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Monocytes Down-Regulate Platelet Activation Induced By a Collagen Surface

Bertrand Arnaud¹, OLLIVIER Véronique¹, Jamel El Benna², Martine Jandrot-Perrus¹, Ajzenberg Nadine^{1,3}

¹Inserm U698 Paris, France, ²INSERM U773, CRB3, Université Paris 7 Paris, France,

³ Department of Hematology, Bichat Hospital, APHP, Paris, France

Damage of blood vessels exposes the subendothelial matrix and results in the adhesion of platelets and monocytes at the site of injury. We examine reciprocal interactions between platelets and monocytes upon exposure to a collagen surface. Washed platelets and CD14 isolated monocytes were incubated separately or together in a 1/100 ratio in plates coated with type I fibrillar collagen. Platelet activation was assessed by measuring P-selectin expression by flow cytometry and RANTES secretion by ELISA. Platelet adherence and activation on immobilized collagen was analysed by confocal microscopy using FITC-phalloidin. Alternatively, cell-cell contacts were prevented by incubating platelets and monocytes in transwell coculture dishes, both parts of which were coated with collagen. In selected experiments, cells were pretreated with the anti-PECAM 1.3 monoclonal antibody or with L-NMMA (NG-methyl-L-arginine), inhibitor of NO synthesis. We have focused our attention on the effect of monocytes on platelet activation. Unexpectedly, when monocytes were added five minutes after platelets to the collagen-coated plates, we obtained a decreased platelet expression of P-selectin by 42% ($p = 0.0053$, $n = 16$) and RANTES secretion by 39% ($p < 0.0001$, $n = 6$). Platelets incubated with immobilized collagen adhere and formed large aggregates consistent with a strong activation state. When monocytes interact with platelets, the number and size of aggregates were dramatically decreased and isolated platelets were observed. In transwell coculture dishes, platelet P-selectin expression and RANTES secretion returned to the levels obtained in the absence of monocytes indicating that cell-cell contacts were required to inhibit platelet secretion induced by collagen. Preincubation of monocytes with anti-PECAM 1.3 reduced the inhibition of collagen-induced P-selectin expression and of RANTES secretion by ~ 40 %. Moreover, anti-PECAM 1.3 reversed the inhibitory effect of monocytes on platelet aggregates. In the presence of L-NMMA pre-treated monocytes, RANTES secretion was similar to the value obtained in the absence of monocytes. Together, our data provide evidence that, monocytes limit the initial phase of platelet activation by a collagen surface. The mechanism is cell-cell contacts dependent, mediated in part by PECAM-1 with NO contribution.

ABSTRACT 4

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Inhibition of nitric oxide production in human intestinal epithelial cells by enterohaemorrhagic *Escherichia coli* results in increased Shiga-toxin synthesis

Marjolaine Vareille¹, Thibaut de Sablet¹, Anne-Lise Glasser², Christine Martin¹, and GOBERT Alain P.¹

¹Unité de Microbiologie, INRA, Centre de Theix, 63122 Saint-Genès-Champanelle, France.

²Pathogénie Bactérienne Intestinale, USC INRA 2018, Université d'Auvergne, Clermont-Ferrand, France

Background: Enterohaemorrhagic *Escherichia coli* (EHEC) are food borne pathogens causing haemolytic-uremic syndrome. EHEC belongs to different serotypes such as O157:H7 and O113:H21. They express various virulence factors including Shiga-toxin (encoded by the gene *stx* carried by a phage lambda), flagellin (encoded by the gene *fliC*), or effector proteins injected in host cells by a type III secretion system (TTSS). After ingestion, EHEC adhere and interact with intestinal epithelial cells and induce the production of inflammatory mediators. These products may in turn act on bacteria. Nitric oxide (NO) is a free radical which possesses numerous immunological functions. Under pathological conditions, NO is synthesized by the inducible NO synthase (iNOS) that is under the control of transcription factors STAT-1 and NF- κ B. Nonetheless, i) the modulation of iNOS expression in human epithelial cells in response to EHEC, and ii) the effect of NO on EHEC, remain unknown.

Results: Expression of the iNOS gene was not modified in the human epithelial cell lines Hct-8 and Caco-2 infected with O157:H7 and O113:H21 EHEC. iNOS mRNA was upregulated by a STAT-1-dependent pathway in cytokine-activated cells when compared to unstimulated cells. However, when EHEC strains were added to cytokine-treated cells, STAT-1 DNA-binding activity, iNOS mRNA expression, and NO production were suppressed. NF- κ B activation was not modulated by cytokines nor by EHEC. The inhibitory effect on iNOS induction was observed when bacteria were separated from the cells by a filter support, when bacteria supernatants were used, and when cells were infected with mutant strains lacking *eae* (adhesion factor), *espA* (TTSS), *fliC*, or *stx*. In addition, NO released from chemical NO donor or from activated Hct-8 cells inhibited i) the synthesis of the phage carrying the gene *stx*, ii) *stx* mRNA expression and iii) Stx production, without modification of bacterial survival.

Conclusions: EHEC release factor(s) inhibiting STAT-1 signalization and iNOS transcription in human epithelial cells. NO is an unrecognized inhibitor of Stx production by EHEC. Therefore, our results highlight a new strategy elaborated by EHEC to escape the host innate immune response and to favor their own Stx production, leading to the development of haemolytic syndromes.

ABSTRACT 5

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SESSION 3

Myeloperoxidase as a mediator in inflammatory disease.

Wanda Reynolds (Sidney Kimmel Cancer Center, San Diego)

Mounting evidence implicates myeloperoxidase (MPO) in chronic inflammatory diseases. MPO catalyzes a reaction between chloride and hydrogen peroxide to produce the potent oxidant hypochlorous acid (HOCl), while reacting with nitrite and NO to generate reactive nitrogen intermediates. MPO plays a protective role in the innate immune response against invading microbes, yet the inadvertent induction of MPO expression by oxidized lipids at atherosclerotic lesions, or by amyloid deposits in Alzheimer's disease, is pathogenic. These positive and negative impacts have led to a complex mechanism of MPO gene regulation. The human MPO gene is regulated by a battery of nuclear receptors, including PPAR γ , LXR, and estrogen receptor (ER), as well as statins and IFN γ /LPS. An MPO promoter polymorphism, -463G/A, results in higher expression for the MPOG allele, correlating with SP1 binding, while the lower expressing MPOA allele preferentially binds ER. In case-control studies, this polymorphism is associated with risk in many inflammatory disease states, including atherosclerosis, Alzheimer's, cystic fibrosis, and ANCA-vasculitis. The -463G/A polymorphism is in an upstream Alu element, within a cluster of receptor binding sites. PPAR γ binds this region and strongly induces MPO expression in MCSF-macrophages, while repressing MPO in GMCSF-macrophages. Estrogen receptor blocks the actions of PPAR γ , especially on the MPOA allele with the stronger ER binding site. The mouse MPO gene lacks the primate-specific Alu, and is not induced by PPAR γ in macrophages. To facilitate the use of mouse models of inflammatory disease, we generated transgenic mice expressing the human MPO G and A alleles. The huMPO transgenics, crossed to atherosclerosis-prone LDLR deficient mice and fed a high fat diet, develop more extensive atherosclerotic lesions, correlating with higher serum cholesterol and obesity. MPO-oxidation of lipid transport molecules such as apoA-1 may underlie the observed hyperlipidemia. MPO also contributes to the regulation of iNOS gene expression in macrophages. The mechanism involves the consumption by MPO of low levels of NO which are required for early events in iNOS induction by IFN γ /LPS. Further understanding the regulation of MPO gene expression, and the role of MPO in iNOS induction, may suggest new pathways for alleviating chronic inflammatory disease.

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Exposure of alveolar and bronchial epithelium to myeloperoxidase modulates epithelial cell responses to pro-inflammatory stimuli

HAEGENS Astrid, Juanita H.J. Vernooy, Peter Heeringa*, Brooke T. Mossman and Emiel F.M. Wouters

Department of Respiratory Medicine, University Hospital Maastricht, Maastricht, The Netherlands, Department of Pathology and Laboratory Medicine*, University Medical Center Groningen, Groningen, The Netherlands and Department of Pathology#, University of Vermont, Burlington, VT 05405 USA

Myeloperoxidase (MPO) is a peroxidase stored in granula of neutrophils. It is released during inflammation and causes an oxidative burst, killing invading micro organisms. Recent observations suggest that MPO also has pro-inflammatory properties, independent of its catalytic activity. In case of uncontrolled inflammation, neutrophils undergoing secondary necrosis release defence enzymes, including MPO, which possibly damage resident lung cells. The aims of this study were to characterize MPO up-take in lung epithelial cells and to investigate the effect of MPO on oxidative stress, DNA damage and interleukin (IL)-8 production by lung epithelial cells under basal and inflammatory conditions.

Human alveolar (A549) and bronchial (Beas-2B) epithelial cells were stimulated with MPO with or without priming the cells for 24 hours with lipopolysaccharide (LPS), Phorbol 12-myristate 13-acetate (PMA) or asbestos, to mimic a pro-inflammatory state. MPO protein was detected in cell cytoplasm with western blot (WB). mRNA levels of heme-oxygenase I (HO-I), an oxidative stress marker, was determined by qPCR. DNA strand breakage was determined using COMET assay. The production of the neutrophil chemo attractant IL-8 was determined by ELISA and transcriptional activity of NF- κ B and AP-1, transcription factors controlling IL-8 expression, were determined with WB.

WB analyses of MPO stimulated A549 and Beas-2B cells demonstrated that MPO was present in the cytoplasm 30 min after stimulation, but did not accumulate. HO-I expression was increased after MPO stimulation and increased further when cells were primed before MPO stimulation. Furthermore, MPO exposure induced DNA strand breakage. Interestingly, MPO inhibited IL-8 production by Beas-2B cells after priming with PMA. This effect was not seen after priming with other mediators and was absent in A549 cells. However, transcriptional activity of NF- κ B and AP-1 was not decreased by MPO stimulation.

In conclusion, alveolar and bronchial epithelial cells are potential targets for MPO. Stimulation with MPO increases HO-I mRNA expression and DNA strand breakage, suggesting that MPO is capable of damaging lung epithelial cells. In addition, MPO inhibition of PMA induced IL-8 production by Beas-2B cells indicates that MPO may be involved in a negative feedback loop for the recruitment of neutrophils.

ABSTRACT 6

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Nitrosylated N-telopeptide of type III collagen (IINys): A new specific biochemical marker of oxidative-induced synovial tissue metabolism in arthritis

CHARNI-BEN TABASSI Nadine¹, P. Richardot¹, C-Bay-Jensen², J-M Délaissé², P. Garnero³

¹Molecular Markers, SYNARC, LYON, FRANCE; ²Clinical Research Unit/Dept. of Clinical Cell biology, Center for Health Science Research and Education, Vejle Hospital, Denmark; ³INSERM U 664, Lyon cedex 03.

Background: Nitric oxide (NO) is an important mediator of joint inflammation and destruction in rheumatoid arthritis (RA) and osteoarthritis (OA). Peroxynitrite induced by NO can react with amino acids including tyrosine (Y) to form nitrotyrosine. Increased NO-related species have been found in the joint of patients with OA or RA, especially in synovial tissue. The aim of this study was to develop an immunoassay recognizing nitrosylated N-telopeptide of type III collagen -one of the main constituent of synovial membrane- to monitor oxidative-related joint damage in arthritis.

Patients and Methods: We produced a polyclonal antibody raised against a nitrosylated sequence specific of the N-telopeptide of human type III collagen (IINys). Using IINys antibody, we performed immunohistochemistry of synovial tissue from 12 patients with knee OA undergoing total joint replacement. We also developed a competitive ELISA to measure IINys levels in the synovial fluid, serum and urine of healthy controls and patients with OA or RA.

Results: The IINys antibody did not recognize the non-nitrosylated sequence of type III collagen N-telopeptide, nitrosylated BSA and free nitrotyrosine, indicating high specificity for both nitrosylation and type III collagen sequence. Immunohistochemistry of synovial tissue from patients with knee OA, showed strong IINys staining in the extracellular matrix, particularly around the synoviocytes and within macrophage-like cells. The ELISA for serum IINys demonstrated intra and inter-assay CV below 15% and recovery of diluted serum samples ranged from 96.6 to 118.3% (mean: 99%). Detectable levels of IINys were measured in the synovial fluid, serum and urine of 12 patients with knee OA. Compared to 30 healthy postmenopausal women, serum IINys levels were increased by an average of 195% ($p < 0.0001$) in 30 postmenopausal women with early RA.

Conclusion: We have developed an immunoassay which detects specifically nitrosylated type III collagen N-telopeptide (IINys). The strong immunoreactivity of IINys in the synovial tissue of patients with OA and the marked increased serum IINys levels in patients with RA, suggest that this new biochemical marker should be useful for the investigation of oxidative-induced alterations of synovial tissue in patients with RA or OA.

ABSTRACT 7

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Sulphur mud-bath therapy in treatment of osteoarthritis: possible antioxidant role

COSTANTINO Maria^{1,2}, X Norel³, C Brink³, G. Nappi⁴, E. Lampa², F Rossi², A Filippelli²

1. CE.R.I.S.T. srl- CENTRO RICERCHE E STUDI TERMALI-NAPLES-ITALY;
2. Department of Experimental MedicinePharmacological Division, II University of Naples-Italy;
3. INSERM U698, Haemostasis, Bio-engineering and Cardiovascular Remodelling. CHU X. Bichat, secteur Claude Bernard - Paris France.
4. Department of Thermal (Spa) Therapy, University of Milano-Italy

Objective: Osteoarthritis (OA) is a degenerative osteoarticular disease that causes the degradation of the articular cartilage, sinovium inflammation and overgrowth of the bone. All these pathological changes produce pain during articular movements, functional difficulty and articular stiffness. Most studies have demonstrated that mud-bath therapy act on the symptomatology of OA, however, the mechanism of action has not been elucidated (Costantino et al., 2002). The literature suggests an increase in the production of TNF- α , IL1, MMP and free radical formation (reactive oxygen metabolites (ROS), or nitric oxide (NO)) during OA. On the basis of these considerations we have evaluated the antioxidant effect of a cycle of mud-bath therapy with sulphur mineral water and safety in subjects suffering of OA.

Materials and Methods: The study was performed on 36 OA subjects as diagnosed according to the American College of Rheumatology criteria. The patients were underwent 12 sessions of mud-bath therapy with sulphur mineral water from Telesse Terme (Benevento-Italy). At the beginning and at the end of the considered treatment we have quantified: the adverse reactions and the serum concentration of ROS [ROS]. Results (mean \pm SD) are expressed in U.Carr. (1 U.Carr. =0.08mg/dl of H₂O₂) The statistic analysis has been performed with Student's t-test with a confidence level of 95%

Results: The results of this preliminary clinical-experimental investigation demonstrate that sulphur mud-bath therapy induced a significant (P< 0.01) reduction of the [ROS] (344 \pm 61 U.Carr. versus 306 \pm 49 U.Carr.). Moreover, at the end of the treatment 53% of the subjects showed [ROS] values in the normal range: 250-300 U.Carr. In addition, no significant adverse reactions have been observed.

Conclusions: Our data show an antioxidant action of sulphur mud-bath therapy in OA. The reduction of the [ROS] could be due to the effectiveness of the mud-bath therapy to reduce the inflammation and the ROS release from the white blood cells as has been reported in the literature (Farber et al., 1990; Shanley et al.,1995; Finch et al., 2004). Considering the fundamental mechanisms of OA the results of this first investigation show that the sulphur mud-bath therapy could be of utility in treatment of these patients.

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SESSION 4

Heme oxygenase: from heme degradation to modulation of redox signaling and inflammation

Jorge Boczkowski (INSERM U700, Paris)

The microsomal enzyme heme oxygenase (HO) catalyzes the oxidation of heme to biliverdin (converted to bilirubin by a biliverdin reductase) and carbon monoxide (CO). HO is widely distributed in mammalian tissues. Three isoforms, products of different genes, have been identified: heme oxygenase-1 (HO-1), the inducible form (also known as heat shock protein 32), and HO-2 and HO-3, the constitutives form. HO-1 expression is extremely sensitive to a variety of agents that cause oxidative stress and/or inflammation.

HO-1 has shown increasing number of antioxidant and anti-inflammatory properties, which confer to the enzyme overexpression protective effects in various models of oxidative injury and/or inflammation (1). The antioxidant effect has been mainly attributed to production of bilirubin, one of the end products of heme catabolism with ROS scavenging properties (2, 3), or to ferritin induction after release of the central iron from heme (4). The anti-inflammatory effect has been attributed to the effect of carbon monoxide (5). However, these effects could be also related to the control of cellular heme content. Indeed, heme degradation is critical for cell homeostasis, because of its potential pro-oxidant effects when it is in the free form (6). Heme availability is also critical for activity of heme-dependent pro-oxidant and/or pro-inflammatory enzymes. Expression and activity of cyclooxygenase-2 (7), nitric oxide synthase (8), or NAD(P)H oxidase (9), three enzymes requiring heme in their catalytic site, is decreased after HO-1-induced heme depletion. Furthermore, HO-1 is expressed not only in microsomes but also in mitochondria, where it controls oxidants production by controlling expression of mitochondrial-located nitric oxide synthase (10). Interestingly, CO can also bind to heme, altering the function of heme-containing enzymes, as demonstrated in the case of nitric oxide synthase or NAD(P)H oxidase (11).

Collectively, these data strongly suggest that the control of the expression and activity of pro-oxidant and/or proinflammatory heme-containing enzymes, secondary to the control of heme availability and/or function, is probably an important mechanism explaining the widespread antioxidant and anti-inflammatory properties of HO-1.

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OXIDATIVE STRESS & INFLAMMATION

January 26, 2007, Pasteur Institute - Paris

Regulation of macroautophagy by NF- κ B transcription factor

DJAVAHERI-MERGNY Mojgan¹, Manuela Amelotti², Julie Mathieu³, Françoise Besançon³, Chantal Bauvy¹, Sylvie Souquère⁴, Gérard Pierron⁴ and Patrice Codogno¹

¹INSERM U756, Faculté de Pharmacie, Université Paris-Sud 11, 92296 Châtenay-Malabry, France. ; ²Laboratory of Biochemistry and Molecular Biology, San Paolo Medical School, 20142 Milan, Italy. ; ³INSERM U685, Centre Hayem, Hôpital Saint-Louis, 75475 Paris Cedex 10, France ; ⁴CNRS-UPR-1983, Institut André Lwoff, Laboratoire de Réplication de l'ADN et Ultrastructure du Noyau, 94801 Villejuif, France.

NF- κ B transcription factors and the signalling that activate them have critical role in cancer development and inflammation responses. Recent evidence has emerge that macroautophagy is another mechanism involved in the regulation of these two processes. Nevertheless, the crosstalk between autophagy and NF- κ B signalling pathways was largely unknown. In the present study, we show that NF- κ B activation mediates repression of autophagy in TNF α treated cells. This repression is associated with an NF- κ B-dependent activation of the autophagy inhibitor mTOR. In contrast, in cells lacking NF- κ B activation, TNF α treatment upregulates the expression of the autophagy-promoting protein Beclin 1, and subsequently induces the accumulation of autophagic vacuoles. Both of these responses are dependent on reactive oxygen species (ROS) production and can be mimicked in NF- κ B-competent cells by the addition of H₂O₂. Small interfering RNA-mediated knock down of beclin 1 and atg7 expression, two autophagy-related genes, reduced TNF α -and ROS-induced apoptosis in cells lacking NF- κ B activation and in NF- κ B-competent cells, respectively. Overall, these results support that repression of autophagy may represent a novel antiapoptotic function of NF- κ B . We suggest that stimulation of autophagy may be a potential way bypassing the resistance of cancer cells to anticancer agents that activate NF- κ B.

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OXIDATIVE STRESS & INFLAMMATION

January 26, 2007, Pasteur Institute - Paris

Pharmacological Induction of Heme Oxygenase-1 Decreases the Acute Phase of Inflammatory Arthritis

BENALLAOUA Mourad^{1,2}, M François^{1,2}, F Batteux^{2,3,4}, J-J Shyy⁶, C Fitting⁷, L Tsagris^{1,2}, J Boczkowski^{8,9}, JF Savouret^{1,2}, MT Corvol^{1,2}, S Poiraudau^{1,2,5}, and RANNOU François

¹INSERM, U747, Laboratoire de Pharmacologie, Toxicologie et Signalisation Moléculaire, Paris, F-75006 France; ²Université Paris Descartes, Paris, F-75006 ; ³CNRS, UPRES 1833, Paris, F-75014; ⁴AP-HP, Groupe Hospitalier Cochin, Laboratoire d'Immunologie, Paris, F-75014; ⁵AP-HP, Groupe Hospitalier Cochin, Service de rééducation, Paris, F-75014; ⁶University of California, Division of Biomedical Sciences, Riverside, CA 92506-0121, USA; ⁷Institut Pasteur, Unit Cytokines and Inflammation, Paris, F-75015; ⁸INSERM, U-408, Paris F-75018 ; ⁹Université Paris7-Denis Diderot, Faculté de Médecine Xavier Bichat, Paris, F-75018

Objective. The objective of the present study was to determine, in vivo, in the acute phase of a non-immune mouse arthritis model, the consequences of 1) Heme Oxygenase-1 (HO-1) pharmacological upregulation and 2) inhibition of HO-1 by injection of a specific anti-HO-1 small interfering RNA (siRNA).

Methods. The K/BxN mouse model of induced arthritis mimics human inflammatory arthritis without lymphocytic influence. In this model, HO-1 was upregulated by intraperitoneal injection of cobalt protoporphyrin IX (CoPP), a potent pharmacological inducer, and inhibited by a specific siRNA. The clinical evaluation of arthritis was monitored by paw thickness. Interleukin-1beta (IL-1beta), IL-6, and tumor necrosis factor-alpha (TNF-alpha) levels, prostaglandin E2 (PGE2) production and metalloproteinase-9 (MMP-9) activity were evaluated in serum. At the end of the experiments, joints were examined for histopathologic changes.

Results. Intraperitoneal injection of CoPP alleviated disease symptoms such as joint swelling, cartilage degradation, and joint inflammatory tissue proliferation in the acute phase of inflammatory arthritis. The CoPP-induced expression of HO-1 in joints and liver was associated with a marked decrease in IL-1beta, IL-6, and TNF-alpha levels; PGE2 secretion; and MMP-9 activity in serum. Conversely, specific HO-1 inhibition by in vivo delivery of the anti-HO-1 siRNA repressed these protective effects.

Conclusion. Our data suggest for the first time that the pharmacological-induced upregulation of HO-1 triggers a robust protective anti-inflammatory response in a non-immune mouse arthritis model. This suggests that exogenously-induced HO-1 may be a potential therapy against the acute phase of inflammatory arthritis in humans.

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OXIDATIVE STRESS & INFLAMMATION

January 26, 2007, Pasteur Institute - Paris

Carbon monoxide generated by heme oxygenase-1 activity confers tolerogenic capacity to dendritic cells

RÉMY Séverine¹, R. Brion¹, P-J. Royer², M. Hill¹, S. Tanguy-Royer², L. Tesson¹, R. Motterlini³, R. Foresti³, M. Gregoire², I. Anegon¹, C. Chauveau¹

¹INSERM U643-ITERT, Nantes, France ; ²INSERM U601, Nantes, France ; ³Northwick Park Hospital, Harrow, UK

Heme oxygenase -1 (HO-1) is the inducible heme oxygenase that catabolizes the degradation of heme into biliverdin, free iron (Fe 2+) and carbon monoxide (CO). Biliverdin is subsequently reduced into bilirubin by biliverdin reductase, and Fe²⁺ induces the expression of ferritin.

Dendritic cells (DCs) play a key role in the immune response and are also increasingly viewed as mediators of T-cell tolerance. We have recently described for the first time that immature DCs express HO-1 and that the expression of HO-1 confers tolerogenic capacity to DCs. We now demonstrate that treatment of human monocyte-derived DCs by exogenous CO [using a CO-releasing molecule (CORM)] blocks LPS-induced increases in MHC II, CD83, CD80, CD86 expression and pro-inflammatory IL-12 cytokine production associated with DC maturation, while preserving the expression of the anti-inflammatory cytokine IL-10. CO treated DCs also display a reduced capacity to induce T cell allogeneic proliferation in vitro. In contrast, treatment of DCs with the other end products of heme degradation by HO-1 (bilirubin, biliverdin and deferoxamine which mimics the action of ferritin) has no effect on DC phenotype or cytokine production. These observations suggest that the effect of HO-1 on DC function is mediated through CO. Interestingly, expression of HO-1 and CO treatment of DCs inhibit the activity of the immunosuppressive enzyme indoleamine 2,3 -dioxygenase (IDO), suggesting that IDO and HO-1/CO may promote immunological tolerance by DCs in distinct contexts.

In conclusion, we show the capacity of CO generated by HO-1 activity to block DC maturation and to inhibit pro-inflammatory and allogeneic immune responses while preserving IL-10 production. This novel immune function for CO may be of interest for the inhibition of immune responses in autoimmune diseases, transplantation, and other conditions involving activation of the immune system.

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January 26, 2007, Pasteur Institute - Paris

Upregulation of nitric oxide in the KNEE-joints of rheumatoid arthritis patients: contribution to a neurogenic pathway of inflammation

WINYARD Paul G*, Meg C O'Shaughnessy#, Claire A Davies#, Bruce L Kidd#, Richard Haigh*, Nigel Benjamin*

*Peninsula Medical School, Universities of Exeter and Plymouth, Exeter EX1 2LU, UK and #Barts and The London, Queen Mary's School of Medicine and Dentistry, London EC1M 6BQ, UK

Nitric oxide (NO) production is increased at inflammatory sites, due to the induction of inducible nitric oxide synthase (iNOS). In rheumatoid arthritis (RA), TNF-alpha appears to be a key "driver" of inflammation, including iNOS upregulation. However, neuropeptides (e.g. substance P; SP) released from nerve fibres in the synovium, as well as infiltrating inflammatory cells, may also contribute to joint inflammation. SP acts primarily through the neurokinin (NK-1) receptor.

We measured the levels of S-nitrosothiols (RSNOs; as a marker of NO) in knee-joint synovial fluid and plasma from patients with RA. We also investigated the role of SP in regulating NO production in the RA joint.

Electron paramagnetic resonance (EPR) spectrometry was used to measure RSNOs in RA plasma and paired synovial fluid (SF), as well as plasma from healthy human subjects. The assay involved degrading RSNOs, and spin trapping the released NO using the Fe(II)-(MGD)₂ complex. RSNOs were significantly elevated in RA SF (median 309 nM) compared with RA plasma, and in RA plasma compared with normal plasma (in which RSNOs were often undetectable). The raised levels of RSNOs in RA SF correlated with some established markers of inflammation. We also examined the capacity of SP to induce NO in both RA fibroblast-like cells and in an experimental model of inflammation. In cultured synoviocytes, not only TNF-alpha and IL-1beta, but also SP, induced significantly increased nitrite concentrations (another marker of NO). In parallel studies, footpad inflammation was induced in NK1 receptor knock-out (KO) and wild-type (WT) mice, and swelling and NO metabolite levels were measured. Plasma NO in WT mice was significantly increased at 3 days following induction of inflammation, but there was no significant change in KO mice. These results were paralleled by the changes in footpad swelling.

In conclusion, we have shown that: (1) S-nitrosothiols, a marker of NO, are increased in SF of RA patients; (2) the upregulation of NO generation within the inflamed joint may be part of a neurogenic pathway of inflammation.

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January 26, 2007, Pasteur Institute - Paris

Plasma antioxidant capacity and CRP values in diabetic foot patients

M. Mohora, B. Virgolici, I. Stoian, A. Coman, L. Gaman, D. Lixandru, V. Gruia, ARAPU Oana

Carol Davila University of Medicine and Pharmacy Bucharest

Plasma C reactive protein (CRP) concentrations correlate with the extent and severity of inflammation. During this process, the blood antioxidant defence systems try to counter the increase in oxygen metabolites. Diabetic patients with high values of CRP have increased vascular risk. Antioxidant therapy was proposed to prevent cardiovascular events but conflicting results were obtained. The aim of this study was to assess the relationship between CRP plasma levels and blood antioxidant capacity in diabetic foot patients.

Forty newly hospitalized diabetic foot patients, aged between 45-75, with different stage foot ulcerations were enrolled. Severity of neuropathy and vascular disease were assessed. Considering CRP values, patients were divided in two groups. Group 1 (n=17) had CRP concentrations between 0,1-1 g/L and group 2 (n=23) had CRP values above 1 g/L. Blood samples were also collected from twenty healthy controls, age and sex-matched. HPLC method was used for glycated hemoglobin and spectrophotometric methods were applied for the other oxidative stress markers. Plasma concentrations for dROM (determinable reactive oxygen metabolites), CRP, ceruloplasmin, dicarbonyls, uric acid and blood glutathion were higher in diabetic patients vs. controls. Plasma thiols and plasma total antioxidant capacity were not significantly modified. Comparing the diabetic patients, plasma concentrations of dROM, ceruloplasmin, uric acid and glycated hemoglobin were significantly increased in group 2 vs. group 1, while plasma thiols level was significantly decreased. Blood glutathion, plasma dicarbonyls and total antioxidant capacity values were similar in both groups. Correlations between plasma dROM and ceruloplasmin and also between uric acid and glycated hemoglobin were calculated for both groups.

These findings indicate that diabetic foot patients with CRP concentrations superior to 1 g/L have an increased plasma oxidative stress. Further studies are necessary to investigate if therapeutic antioxidant intervention should take into account CRP plasma values so that wound healing could be improved.

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January 26, 2007, Pasteur Institute - Paris

Cytokines modulate intracellular oxidant activity in C2C12 myotubes

ARBOGAST Sandrine¹, Jeffrey D Smith², Jackie L Smith² and Michael B Reid²

¹Inserm U582 - Institut de Myologie-G.H. Pitie-Salpetriere - 75 561 Paris Cedex 13 - France

²University of Kentucky, Department of Physiology, MS-509 Chandler Medical Center- Lexington KY 40502 USA

Nitric oxide (NO) and reactive oxygen species are produced in pathological conditions (sepsis, diabetes, and COPD). These illnesses are associated with an increase in circulating cytokines. This study examines the effect of different cytokines on intracellular oxidant activity. We hypothesized that 1) Cytokines will increase oxidant activity in C2C12 myotubes 2) Interferon gamma (IFN γ) will potentiate the effect of tumor necrosis factor alpha (TNF α) increasing NO production after 48h. We measured the overall oxidant activity using DCFH-DA and NO derived activity using DAF-FM. C2C12 myotubes were treated for 1 hour with TNF α or IFN γ . In the second protocol, we examined the effect of repeated treatments with TNF α , IFN γ or TNF α + IFN γ after 48hours. iNOS expression was measured using RT-PCR, and western blot. After 1 hour, TNF α increased DCFH sensitive oxidant activity (+ 155%, P< 0.05) whereas IFN γ had no effect. After 48h, TNF α or IFN γ increased overall oxidant activity. TNF α + IFN γ was the only treatment that significantly increased DAF signal (+ 190%, P< 0.05). The signal was abolished using the NOS inhibitors L-NAME and 1400W. This result was corroborated by an increase in iNOS mRNA expression (+600 fold, P<0.05) and iNOS protein (+15876%, P<0.05). These data indicate that different oxidant species are involved in cytokine - induced response.

Supported by NIH grant HL 59878.

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OXIDATIVE STRESS & INFLAMMATION

January 26, 2007, Pasteur Institute - Paris

NADPH-oxidase involvement in phosphatidylserine exposure in neutrophils

BOURGE Mickaël, Doignon Isabelle, Nüsse Oliver, Sulpice Jean-Claude

INSERM UMR-S757, Signalisation calcique et interaction cellulaires dans le foie, Université Paris Sud XI - 91405 Orsay

NADPH-oxidase from neutrophils produces reactive oxygen species (ROS), which contribute to their destruction power against pathogens. Neutrophil activation also regulates their own apoptosis, which enables them to limit their action. ROS production maybe involved in the induction of apoptosis. ROS production by NADPH-oxidase is coupled to intracellular proton release of which the effects on cellular death are poorly understood. Oxidative processes and acidification are controlled by cellular protective systems (oxidant catabolism, proton channels and sodium-proton exchanger (NHE)). Therapeutic induction of neutrophils apoptosis is a challenge in treatment of chronic inflammations. Early exposure of phosphatidylserine (PS) is involved in the elimination of apoptotic cells by macrophages, which contributes to repress inflammation. The goal of this study is to characterize the impact of NADPH-oxidase activation (ROS production and acidification) on the PS exposure process.

In human myeloid cells (PLB-985) differentiated into neutrophils, phagocytosis of opsonized zymosan or PMA treatment induced PS exposure, which was drastically inhibited, but not fully suppressed by DPI as well as in cells deficient in NADPH-oxidase. The data suggest a preponderant, but not exclusive role of the oxidase in PS exposure. Elimination of ROS by exogenous reducers did not affect PS exposure, excluding a direct implication of oxidative processes. PMA or phagocytosis induced a limited cytosolic acidification, drastically increased in presence of NHE inhibitors, suggesting an important role of the exchanger in the proton elimination. Inhibition of NHE significantly increased PS exposure, which was correlated with the intracellular acidification. Only the number of PS molecules per cell was increased without changing the proportion of responding cells. Nevertheless a direct acidification induced in un-activated cells had no effect on PS exposure.

Altogether, these data allow us to propose inhibition of NHE as a target to promote elimination of activated neutrophils in inflammatory diseases.

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OXIDATIVE STRESS & INFLAMMATION

January 26, 2007, Pasteur Institute - Paris

Oral contraception and lipid peroxidation

de GROOTE Donat PhD, Joël Pincemail PhD*, Jean-Olivier Defraigne MD* and Jean-Michel Foidart MD*****

* Université de Liège, Service de Chirurgie Cardiovasculaire et CREDEC, CHU Sart-Tilman, B-4000 Liège, Belgique,

** PROBIOX SA, Campus Universitaire du Sart-Tilman, Avenue de l'hôpital, Tour GIGA, Bât.B34, B-4000 Liège,

Belgique, *** Université de Liège, Département de Gynécologie et Obstétrique, Sart-Tilman, B-4000 Liège, Belgique

Objectives. Evaluation of the influence of oral contraception with estrogens and progestins on several markers of the oxidative stress.

Material and methods. A group of 53 women taking oral contraceptives containing estrogens and progestins has been compared to a matched control group of women using no oral contraceptives. The oxidative status included antioxidants, trace elements and oxidative stress markers. Median values of the two groups were compared using appropriate statistical methods.

Results. Levels of zinc were slightly lower but copper, copper to zinc ratio and lipid peroxides significantly higher in the group taking the contraceptive pill compared to the control group. There was a significant positive correlation between the lipid peroxide levels and the copper to zinc ratio.

Conclusions. We showed a significant relationship between the use of oral contraceptives of the last generation and the presence of heightened lipid peroxidation that is thought to be associated with increased cardiovascular risk. In view of these results, it seems to us that the establishment of oxidative stress status would allow doctors to propose adapted and personalized contraception solutions to their patients.

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January 26, 2007, Pasteur Institute - Paris

Mechanisms of oxidative stress-induced apoptosis of fibroblasts in response to 5-aminolaevulinic acid photodynamic therapy

DOGRA Yuktee, Alison Curnow and Paul Winyard

Peninsular College of Medicine and Dentistry, IBCS, St Luke's Campus, Magdalene Road, Exeter, Devon, EX1 2LU

A key feature of the inflammatory response in the joints of patients with rheumatoid arthritis (RA) is the proliferation of synovial fibroblasts involved in the formation of an invasive 'pannus' tissue. This pannus appears to mediate the destruction of cartilage and bone. In relation to treatment, the approach of obliterating the pannus tissue by surgical synovectomy or by 'chemical synovectomy' has been investigated. A further experimental extension of the chemical synovectomy approach has been to carry out photodynamic therapy (PDT) synovectomy including 5-aminolaevulinic acid (ALA) PDT.

PDT is a highly selective way to ablate tissue. PDT is a ternary treatment involving three key components: a photosensitiser, light and tissue oxygen. The combination of these components causes reactive oxygen species production, which induces cell death via apoptosis and/or necrosis. In experimental protocols using in vivo models of inflammatory joint disease ALA is administered intravenously and is then naturally converted into the photosensitiser, protoporphyrin IX (PPIX). This method is a less invasive way of ablating inflamed synovial tissue compared to a surgical synovectomy.

The current study investigates the molecular mechanisms of ALA-PDT induced apoptosis in fibroblasts, assessing the amount/type of cell death occurring and also the reactive oxygen species being produced. This is with a view to enhancing treatment protocols. Fibroblasts (84BR) were exposed to 0.5 mM ALA, six hours before irradiation with a non-coherent, xenon arc lamp (630 ± 15 nm; Paterson) with a fluence of 0.08 W/m² for 5 minutes. The cells were assessed for cell death 16 hours after irradiation by annexin V binding and fluorescence microscopy. ALA-PDT exposed cells exhibited apoptosis in 46 ± 3.9% (mean ± 1SD) of the cells counted. Cells treated with ALA in the absence of light irradiation exhibited 20 ± 3.7% apoptosis. All other control groups showed % apoptosis similar to this latter value. The PDT exposed population therefore demonstrated a statistically significant increase in % of apoptosis compared to the non-exposed control groups (p<0.01; Student's t-test). This in vitro cell model will be further employed to explore the effects of pro- and antioxidant agents.

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January 26, 2007, Pasteur Institute - Paris

UCP2 modulates LPS-induced MAPK signaling in macrophages

EMRE Yalin, Hurtaud C., Nubel T., Criscuolo F., Ricquier D., Cassard-Doulcier A.M.

CNRS UPR 9078, Faculte de Medecine Paris 5 – Necker - 75730 Paris, France

The mitochondrion is a major organelle contributing to energy metabolism but also a main site of reactive oxygen species (ROS) production. Lipopolysaccharide (LPS)-induced ROS signaling is a critical event in macrophage activation. We report here that part of LPS-mediated ROS signaling comes from mitochondria inside a signal amplification loop that enhances mitogen-activated protein kinase (MAPK) activation. More precisely, we identified the inner mitochondrial membrane uncoupling protein 2 (UCP2) as a physiological brake on ROS signaling. Stimulation of murine bone marrow-derived macrophages by LPS quickly downregulated UCP2 through the JNK and p38 pathways. UCP2 downregulation was shown to be necessary to increase mitochondrial ROS production in order to potentiate MAPK activation. Consistent with this, UCP2-deficient macrophages exhibit an enhanced inflammatory state characterized by increased nitric oxide production and elevated migration ability.

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January 26, 2007, Pasteur Institute - Paris

Structural organization of the entire p47phox protein brings new perspective into the molecular mechanism of the neutrophil NADPH oxidase activation

D. Durand, D. Cannella, Virginie Dubosclard, E. Pebay-Peyroula, P. Vachette, and FIESCHI Franck

Institut de Biologie Structurale, 41 rue Jules Horowitz - 38027 Grenoble

In response to microbial infection, neutrophils promote the assembly of the NADPH oxidase complex in order to produce superoxide anions during the inflammation. This reaction is activated by the association of the cytosolic factors, p47phox, p67phox, p40phox and a small G protein Rac with the membranous heterodimeric flavocytochrome b558, composed of gp91phox and p22phox. In the activation process, p47phox plays a central role as the target of phosphorylations and as a scaffolding protein conducting the translocation and assembly of cytosolic factors onto the membranous components. The PX and tandem SH3s of p47phox have been highlighted as being key determinants for the interaction with membrane lipids and the p22phox component respectively. In the resting state, the two corresponding interfaces are thought to be masked allowing its cytoplasmic localization. However, the resting state modular organisation of p47phox and its auto-inhibition mode are still not fully understood despite available structural information on separate modules. To address this question we have engaged a study of p47phox in solution using Small Angle X-ray Scattering. Even in the resting state, despite internal auto-inhibitory interactions, p47phox adopts an extended conformation. First insights about the domain arrangement in whole p47phox can be derived. Our data allow to discard the usual representation of a globular and compact auto-inhibited resting state and open new perspective into the molecular mechanism of the NADPH oxidase activation in response to microbial infection.

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OXIDATIVE STRESS & INFLAMMATION

January 26, 2007, Pasteur Institute - Paris

Inflammatory response induced by Diffusely Adhering Escherichia coli harboring Afa/Dr adhesins (DAEC Afa/Dr)

GLEIZES Aude*§, C. Sandré*, I. Turbica*, N. Sémiramoth*, I. Kansau*, C. Berger*, R. Gorges-Kergot*, A. Servin* and S. Chollet-Martin* #

*INSERM U756, IFR 141, Faculté de Pharmacie de Paris Sud 11, Chatenay-Malabry; §; Service d'Immunologie Biologique, CHU Henri Mondor, Créteil; # Service d'Immunologie Biologique, CHU Bichat, Paris

Diffusely Adhering Escherichia coli harboring Afa/Dr adhesins (DAEC Afa/Dr) have been found to be associated not only with urinary and enteric infections but also with inflammatory bowel diseases. The aim of this study was thus to develop an in vitro model of interactions between these bacteria, intestinal epithelial cells and neutrophils. The human myeloid leukaemia cell line PLB-985 differentiated in terminally mature neutrophils was used to develop this model. Using flow cytometry, confocal microscopy and western blot we evidenced the expression of some DAEC Afa/Dr receptors mainly Carcinoembryonic Antigen-related cellular Adhesion Molecule 6 and DAF (CD55) as well as receptors involved in epithelial interactions (CD11b, DAF-L). The functional effects of 2 wild-type DAEC strains (IH11128, C1845) differing by their adhesive properties (Dr or F1845 adhesins expression, respectively) were then carried out on differentiated PLB-985. A non-virulent E. Coli strain (AAEC 185) and its mutants expressing Dr or F1845 adhesins served as controls. LPS was also extracted and purified from the various strains. Oxidative burst was quantified by both dichlorofluorescein diacetate assay (DCFH-DA) measuring hydrogen peroxide (H₂O₂) production by flow cytometry, and cytochrome C reduction assay analysing superoxide anion (O₂^{•-}) production. Pro-inflammatory cytokine (IL-8, IL-1 beta and TNF alpha) production was tested by ELISA. The results show that, among the different E. Coli strains, the wild type IH11128 induced the greatest response in particular reactive oxygen species and IL-8 productions. Moreover, the AAEC185 strains, expressing or not the Dr adhesin, induced lower responses suggesting the implication of other virulence factors such as F1845 adhesin, LPS or toxins. In conclusion, these data suggest the ability of DAEC Afa/Dr to modulate PLB-985 oxidative burst and cytokine production. This model could be useful to study the intestinal inflammatory response induced by such E Coli strains.

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January 26, 2007, Pasteur Institute - Paris

Inhaled oxidation catalysis treated diesel engine emissions induce a marked systemic oxidant stress. Is NO₂ modulation the main trigger ?

HASSON Virginie^{1,2}; Keravec Veronika²; Preterre David²; Lorient Stephane¹; Henry Jean-Paul¹; Dionnet Frederic²; Morin Jean-Paul¹.

¹.U644, INSERM, ROUEN, France.

².CERTAM, SAINT ETIENNE DU ROUVRAY, France

Due to the onset of oxidation catalysis (oxycat) as a Diesel emission after-treatment strategy, the oxidant potential of Diesel emissions increased which can be probed by the NO₂/NO_x ratio which correlates with the amount of reactive oxygen species to be generated from the atmosphere to aqueous media containing CPH as a spin probe and assayed by electron spin resonance (ESR). In this study, we assess the impact of 3 consecutive daily inhalations for 3 hours of diluted untreated (UDE) (low oxidant) and Oxycat treated (ODE) (high oxidant) Euro3 Diesel engine emissions (dilution 1:50, PM=0.5mg/m³, NO₂ 0.4ppmUDE and 4ppm ODE) and air and UDE doped with NO₂ at similar concentrations to those found in ODE (4ppm). Plasma TNF α , and lung and heart SOD and GPx activity levels were assayed in healthy (H) and chronic myocardial infarction (MI). ESR measurements of ROS ranked as: AIR. ESR results show correlation between NO₂ and ROS production with an increasing after oxycat in contrast after untreated emissions. NO₂ alone induced systemic oxidant stress: lung and heart GPx activities increase in both H and MI rats while no impact was seen on SOD. ODE had little impact on GPx activity but for increased levels in MI rat heart, while it provoked increased activities of SOD in both H and MI rat lungs and heart. A moderate impact of UDE+NO₂ was seen on SOD activities in MI lungs and hearts, while no impact was seen in H rats. In both UDE+NO₂ and ODE, impacts on lung GPx appear to be less marked than for NO₂, suggesting potential quenching of NO₂ impact by the co-presence of DE acting on SOD activity. Concerning TNF α , increased serum levels were similar in MI rats after NO₂, UDE+NO₂ and ODE, while more disparate records were found in H rats with again a net tendency to increased levels. Diesel emission treatment with oxycat provokes a marked systemic oxidant stress and elevation of serum TNF α , which cannot be totally assigned to the sole modulation of NO₂ concentrations, other ROS might be involved. MI rats appear to be more sensitive than healthy rats.

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OXIDATIVE STRESS & INFLAMMATION

January 26, 2007, Pasteur Institute - Paris

Inflammation of human preadipocytes induced by macrophage-secreted factors

LACASA Danièle, Keophiphath Mayoura, Henegar Corneliu, Miranville Alexandra, Zucker Jean-Daniel and Clément Karine

INSERM, U755 Nutriomique, University Pierre and Marie Curie-Paris 6, Faculty of Medecine les Cordeliers, AP-HP, Hotel-Dieu Hospital, Nutrition Department, 1 place du Parvis Notre-Dame, 75004 Paris, France

Obesity is now considered a chronic and low-grade inflammation and associates with macrophage infiltration into adipose tissues. These macrophages could constitute an important source of inflammation in expanded adipose tissues. Our working hypothesis stems that macrophage infiltration limits fat expansion through paracrine action on the precursors of adipocytes, the preadipocytes. To test this hypothesis, human preadipocytes were cultured in the presence of conditioned media from activated macrophages differentiated from blood monocytes or macrophages isolated from obese adipose tissue. Preadipocytes treated by macrophage secreted factors displayed an activation of NF-kappaB signaling and increased gene expression and release of pro-inflammatory cytokines (IL6) and chemokines (MCP-1, IL8 and CCL5). Anti TNFalpha neutralizing antibody treatment inhibits preadipocyte inflammatory changes identifying TNF± as a major macrophage-derived mediator of preadipocyte inflammation. To get more insight on signaling pathways mobilized in inflammatory preadipocytes, cDNA microarray analyses were performed to compare gene expression profiles between control and inflammatory preadipocytes. This comparison identified a set of up-regulated genes in inflammatory preadipocytes. Several genes are NF-kappaB targets reinforcing the importance of this pathway in inflammatory preadipocytes. A in house bio-informatic treatment identified enriched functional clusters which best characterized inflammatory preadipocytes. Two of the most enriched functions corresponded to « Extracellular matrix » (ECM) and « Extracellular region » comprising genes with patho physiological interest; notably Fibronectin, its major receptor integrin alpha5 and Tenascin C that were validated at protein levels.

In conclusion, the macrophage-secreted factors induce inflammation changes in preadipocytes with an extensive ECM remodeling. The increased production of cytokines could self perpetuate inflammation within adipose tissue. The chemokines could participate to the recruitment of new macrophages creating then a vicious cycle in this tissue. Thus, macrophage infiltration could represent a non-beneficial adaptative response which limits fat expansion during obesity.

ABSTRACT 22

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OXIDATIVE STRESS & INFLAMMATION

January 26, 2007, Pasteur Institute - Paris

Oxidative stress is an important component of airway inflammation in mice exposed to cigarette smoke

LAGENTE Vincent^{*1}, Jean-Michel Planquois^{†2}, Olivier Leclerc^{†*}, Fabien Schmidlin^{†3} and Claude P. Bertrand^{†4}

^{*1}INSERM U620, Faculté de Pharmacie, Université de Rennes 1, Rennes, F-35043, France; [†]Pfizer Global R&D, Fresnes Laboratories; Fresnes, F-94265 France, ¹Current address: Medical Gases group, Air Liquide - Research Center Claude-Delorme, Jouy-en-Josas France, ²Current address: Eli Lilly, R&D, Indianapolis, IN, USA, ³Current address: Merck sante, Chilly-Mazarin, France, ⁴Current address: AstraZeneca R&D, Alderley Park, Macclesfield, UK.

It has been previously proposed that oxidative stress was a main component of the inflammatory process in chronic obstructive pulmonary disease (COPD). Moreover, cigarette smoke (CS) is the major etiological factor in COPD. To explore the hypothesis that NADPHoxidase-derived oxidative stress generation regulates the inflammation associated with COPD, we investigated the inflammatory response in mice deficient for the p47phox subunit of NADPH-oxidase and exposed them to CS or lipopolysaccharide (LPS) as an experimental model of COPD. Ten-week-old control or p47phox^{-/-} (P47KO) mice were exposed to the whole smoke of 2 cigarettes (Kentucky 1R3 cigarettes) twice a day for 3 days per week for 1, 2, 3, 4, or 12 weeks. In another set of experiments, mice were exposed for 1 hour to 100 µg/ml LPS aerosol. Bronchoalveolar lavages (BAL) were performed thereafter. Differential cell counts as well as the amount of IL-6, KC/CXCL1 and MCP1/CCL2 were analysed in the BAL fluids. Exposure of both control and P47KO mice to CS elicited an increase in the number of macrophages and neutrophils in BAL fluid, which peaked in both groups 3 weeks after the beginning of the experiment. However, BAL fluid of the P47KO mice contained significantly fewer macrophages and neutrophils than that of control mice at 3 and 4 weeks after the experiment began. We also observed that the levels of IL-6, KC/CXCL1 and MCP1/CCL2 measured at 1 to 3 weeks were significantly lower in the BAL fluids of P47KO mice exposed to CS as compared to control mice. Measured 24 h after LPS exposure, the number of macrophages, neutrophils and KC/CXCL1 levels in BAL fluids had increased significantly in both groups of mice and were significantly greater in the P47 KO mice than in the control WT mice. Our study clearly showed that mice in which the P47phox sub-unit of NADPH-oxidase has been deleted, fail to develop airway inflammation after CS exposure. In contrast, the inflammatory response was exacerbated in P47KO mice following LPS inhalation. This suggests that oxidative stress is involved in the pathogenesis of COPD and supports the development of antioxidant therapy for smoking-related lung disease.

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January 26, 2007, Pasteur Institute - Paris

Cross talk between kinase activation / oxidative stress in human dendritic cells maturation induced by contact sensitizers

MIGDAL Camille¹, S. Trompezinski¹, K. Arab², J-P. Steghens² and M. Serres¹

¹EA37-32, Université Claude Bernard Lyon 1 (UCBL1), Pavillon R, Hôpital E. Herriot, Lyon, France; ²EA30-90 UCBL1, Fédération de Biochimie, Hôpital E. Herriot, Lyon, France

Although the maturation of dendritic cells (DC) induced by contact sensitizers is well characterized by a change in phenotype and cytokine production, earlier events involved in this process required to be more specified. Recently, only few studies were reported demonstrating the role of oxidative stress and p38 kinase activation in DC maturation. The cross talk between kinase activation/oxidative stress induced by allergens have to be clarified for a new approach to discriminate sensitizers from irritants. To address this purpose, the role of oxidative stress and cell signaling induced by allergens was evaluated on the maturation of DC derived from human monocytes cell lines (THP1 and U937) which similarly respond to allergens as mono-DC, and prevent from inter-individual variations. Kinase activation was assessed using specific inhibitors and intracellular phosphoprotein analysis by flow cytometry. The role of kinases upstream and downstream p38 MAPK, respectively ASK1 and HSP27, will be investigated as well as PKCdelta which plays an essential role in DC maturation. To confirm their specific role, RNAi specific for these kinases will be investigated. Oxidative stress was detected by flow cytometry using a H2DCF-DA fluoroprobe for ROS and DSSA for glutathione measurement in addition to HPLC-MS. Preliminary data showed that an oxidative stress was induced essentially by 2,4-dinitrochlorobenzene and thimerosal but not by nickel sulfate, in correlation with a specific activation of p38 MAPK and ASK1. These studies will allow to better manipulate DC in inflammatory diseases and for their use in cancer vaccinology.

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January 26, 2007, Pasteur Institute - Paris

Sevelamer Prevents Uremia-Enhanced Atherosclerosis Progression in Apolipoprotein E Deficient Mice

Olivier Phan, MD; Ognjen Ivanovski, MD; Thao Nguyen-Khoa, PharmD, PhD; Nadya Mothu; Jesus Angulo, PhD; Ralf Westenfeld, MD; Markus Ketteler, MD; Natalie Meert, MSc; Julien Maizel, MD; NIKOLOV Igor G, MD; Raymond Vanholder, MD, PhD; Bernard Lacour, PharmD, PhD; Tilman B. Drüeke, MD; Ziad A. Massy, MD, PhD

Inserm U 507, Hôpital Necker – Néphrologie - Paris Cedex 15, France

Background—The novel phosphate binder sevelamer has been shown to prevent the progression of aortic and coronary calcification in uremic patients. Whether it also decreases the progression of atheromatous plaques is unknown. The aim of our study was to examine the effect of sevelamer administration on the development of atherosclerosis and aortic calcification in the uremic apolipoprotein E deficient mouse as an established model of accelerated atherosclerosis.

Methods and Results—Female mice were randomly assigned to 4 groups: 2 groups of nonuremic mice (sevelamer versus control) and 2 groups of uremic mice (sevelamer versus control). Sevelamer was given at 3% with chow. The increases in serum phosphorus concentration and calcium-phosphorus product observed in uremic control mice were prevented by sevelamer. Serum total cholesterol was increased in the 2 uremic mouse groups and remained unchanged in response to sevelamer. After 8 weeks of sevelamer treatment, uremic mice exhibited a significantly lower degree of atherosclerosis ($P < 0.001$) and vascular calcification than uremic control mice. Of interest, sevelamer exerted an effect on both intima and media calcification ($P < 0.005$) in uremic mice. Among possible mechanisms involved, we found no evidence for the modulation by sevelamer of inflammation or selected uremic toxins. In contrast, nitrotyrosine staining as a measure of oxidative damage was significantly decreased in response to sevelamer treatment in control and uremic mice ($P < 0.005$).

Conclusions—Sevelamer delays not only vascular calcification but also atherosclerotic lesion progression in uremic apolipoprotein E deficient mice. It opens the possibility of a cholesterol-independent action of sevelamer on atheroma formation via effects on mineral metabolism, oxidative stress, or both.

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January 26, 2007, Pasteur Institute - Paris

Respiratory dysfunction after local TLR4 but not TLR-2/6 stimulation and the LPS-mediated intravenous escape

NOULIN Nicolas, Isabelle Couillin, Dieudonné Togbé, Bruno Schnyder, Silvia Candrian and Bernhard Ryffel

CNRS, Immunologie et Embryologie Moléculaires - 45071 Orléans la source

LPS and MALP-2 are well known for their role in the induction of inflammation in vivo. They act through TLR-4 and TLR-2/6 respectively, in a MyD88 dependent manner. However, it is unclear whether MyD88 acts synergically with TRIF, another Toll adapter.

In our in vivo experiment we clearly demonstrate a difference between MALP-2 and LPS-induced inflammation. After intranasal application of these TLR agonists in C57/B16 mice, the respiratory function is measured by non-invasive plethysmometry during six hours. Afterwards we perform Broncho-Alveolar Lavage (BAL), search for cytokine production in the airway space and for myeloperoxidase (MPO) activity in lung tissue. Our data show that LPS induces a TLR-4-dependent constriction of the airways whereas MALP-2, a TLR-2/6 agonist, does not cause bronchoconstriction despite similar recruitment of neutrophils in the BAL and in the lung parenchyma.

Due to the fact that bronchoconstriction occurs only 90 minutes after LPS intranasal application, we investigated whether a simultaneous intravenous injection influences the mucosal-induced response. By systemic injection of LPS but not MALP-2 we abrogated respiratory dysfunction induced by local LPS application.

In conclusion, we show that MALP-2 is not able to evoke the airway constriction and that LPS-induced bronchoconstriction can be inhibited by a LPS-intravenous injection while TLR-2/6 systemic administration has no influence on the mucosal effect.

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January 26, 2007, Pasteur Institute - Paris

Phosphorylation of gp91phox/Nox2 in human neutrophils

RAAD Houssam¹, Paquet M-H², Morel F², Quinn MT³, Gougerot-Pocidallo M-A¹, Dang PM¹, and El-Benna J¹

¹INSERM, U773, F-75018, France ; Université paris 7 Denis Diderot, Faculté de Medecine, site Bichat, Paris, F-75018, France ; ²GREPI, EA2938 Université Joseph Fourier, Labo d'enzymologie, 38043 Grenoble, France and ³Department of Veterinary Molecular Biology, Montana State University, Bozeman, MT, USA.

Neutrophils play a key role in host defenses against invading microorganisms and have a major role in inflammation. In response to a variety of agents, they release large quantities of superoxide anion (O₂⁻) and other ROS in a phenomenon known as the respiratory burst. Neutrophil production of O₂⁻ is dependent on activation of NADPH oxidase, a multicomponent enzyme system that catalyzes NADPH-dependent reduction of oxygen to O₂⁻. In resting cells NADPH oxidase is inactive and its components are distributed between the cytosol and membranes. When cells are activated, the cytosolic components (p47phox, p67phox, p40phox and Rac2) migrate to the membranes, where they associate with the membrane-bound component (flavocytochrome b558) to assemble the catalytically active oxidase. Cytochrome b558 is a heterodimeric membranous protein composed of a large subunit (called gp91phox or b-cytb-subunit or Nox2) and a small subunit (p22phox also called a-cytb-subunit). Upon NADPH oxidase activation, p47phox, p67phox, p40phox and p22phox become phosphorylated but the phosphorylation of gp91phox/Nox2 is not defined. In this study, we show that gp91phox is clearly phosphorylated in human neutrophils stimulated with PMA, FMLP, opsonized zymosan and TNF α . Phosphoamino acid analysis showed that the phosphorylation of gp91phox occurred on threonine residues. The PKC inhibitor GF109203X inhibited this phosphorylation, and PKC was able to phosphorylate purified gp91phox in vitro. These results show that gp91phox is phosphorylated in human neutrophils and suggest that PKC is involved in this process. Phosphorylation of Noxs might regulate their activity.

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OXIDATIVE STRESS & INFLAMMATION

January 26, 2007, Pasteur Institute - Paris

Overproduction of IL-8 in CF lung epithelial cells after sodium 4-phenylbutyrate treatment

ROQUE Telma, E. Boncoeur, O. Tabary, E. Bonvin, A. Clement, J. Jacquot

INSERM, UMR S 719, Université Pierre et Marie-Curie-Paris6 ; Hôpital Saint-Antoine, Paris, F-75012 France

Sodium 4-phenylbutyrate (4-PBA), a butyrate analogue that is approved for clinical use in cystic fibrosis (CF) lung disease, has been shown to correct the F508del-CFTR trafficking defect, to restore CFTR function at the plasma membrane of cultured CF lung epithelial cells and to cause a improvement in nasal epithelial chloride transport in F508del-homozygous cystic fibrosis patients. The aim of our study was to gain insights into the potential effects of 4-PBA on the inflammatory response in CF lung epithelial cells. With two CF bronchial epithelial cell types (CFBE41o- and IB3-1 cell lines with F508del-homozygous and heterozygous genotype, respectively), we clearly demonstrated that 4-PBA induced a strong increase of both IL-8 mRNA and protein expression in two CF cell lines whereas no significant variation of IL-1beta mRNA was observed. Unexpectedly, we also reported that treatment of the two cell lines with 4-PBA alone or in combination with 10 ng/ml TNF- α decrease the NF- κ B transcriptional activity and reduced the 20S proteasome activity. These data prompted us to investigate other potential pathways controlling the IL-8 expression by 4-PBA. Inhibition of ERK1/2 signalling pathway by two different inhibitors (U0-126 and PD098059) blocked the increase of IL-8 secretion induced by 4-PBA. These results suggested that 4-PBA mediated IL-8 expression is controlled by ERK-1/2 pathway in an NF- κ B-independent manner. Thus, a combination of 4-PBA treatment with an ERK inhibitor may be beneficial to reduce the lung inflammation in CF patients.

Supported by grants from Inserm, Fondation pour la Recherche Médicale and the French cystic fibrosis association (VLM).

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OXIDATIVE STRESS & INFLAMMATION

January 26, 2007, Pasteur Institute - Paris

Consequences of oxidative stress-induced modifications to the apoptotic bridging molecules in human inflammatory autoimmune disease

RYAN Brent, Dagmara Szeszakowska, Nick Viner, Richard Haigh, Ahuva Nissim, Paul G Winyard, Paul Eggleton

Institute of biomedical and clinical sciences, Peninsula Medical School (St Luke's Campus)
Exeter, EX1 2LU

The first component of complement, C1q, and calreticulin (CRT) are key bridging molecules in the CD91/CRT/C1q pathway of apoptotic cell clearance. These proteins act as bridging proteins, involved in the recognition by phagocytic macrophages of the cell surface of apoptotic neutrophils during the phagocytic clearance of neutrophils.

The oxidative modification of proteins on the cell surface of apoptotic cells has been implicated in the formation of neoantigenic proteins which are recognised by autoantibodies found in patients with autoimmune diseases such as systemic lupus erythematosus (SLE). Autoantibodies to C1q and CRT have been reported in 20% and 50% of SLE patients, respectively. We previously found that 12 out of 24 SLE patients had detectable antibodies to CRT, but there is a literature disparity, in respect of the proportion of SLE patients with anti-CRT antibodies. This may be due to variable levels of CRT modification during the expression/ isolation procedures.

We have investigated the consequences of oxidative modifications to C1q and CRT, in relation to the formation of autoantibodies in SLE patients' sera. We modified C1q using H₂O₂ +/- copper ions, i.e. exposure to a bolus of either H₂O₂ or hydroxyl radicals. In SLE sera, we found increased recognition of IgG by H₂O₂-modified C1q compared with native C1q (2/10 and 9/10 patients' antibodies recognise non-modified C1q and H₂O₂-modified C1q, respectively). In order to study the consequences of oxidative modification to CRT, we established a bacterial expression system for a CRT-thioredoxin (Trx) fusion protein. Trx is a potent, cysteine-containing, antioxidant protein. We employed FPLC to isolate the CRT-Trx fusion protein, as well as the CRT protein obtained by cleaving the fusion protein using thrombin or by isolating native CRT. Further studies will test the consequence for antigenicity of oxidative stress exposure of the CRT-Trx fusion protein compared with the CRT polypeptide alone.

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January 26, 2007, Pasteur Institute - Paris

ATP inhibits the inflammatory response in stimulated whole blood, even under conditions of severe oxidative stress

SWENNEN Els L.R.^{1, 2}, Ilja C.W. Arts¹, Aalt Bast², Pieter C. Dagnelie¹

¹Department of Epidemiology, ²Department of Pharmacology and Toxicology, NUTRIM, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands.

Adenosine 5'-triphosphate (ATP) is a naturally occurring nucleotide found in every cell of the human body. In addition to its well-established intracellular role in cellular metabolism, extracellular ATP exerts pronounced effects in a variety of biological processes such as neurotransmission, muscle contraction and platelet function. Over the past decades, evidence has accumulated which indicates that extracellular nucleotides may be important regulators of inflammatory responses. Many diseases, such as COPD and cancer, in which inflammatory reactions are involved are also associated with oxidative stress. The purpose of the present study was to determine the effects of ATP on the release of the pro-inflammatory cytokines TNF- α and IL-6, and the anti-inflammatory cytokine IL-10 in stimulated whole blood in the absence and presence of oxidative stress.

Blood samples were drawn from 8 healthy volunteers (25-45 yrs of age) and incubated with ATP and lipopolysaccharide + phytohemagglutinin for 24 h, in both the absence and presence of H₂O₂.

In the absence of H₂O₂, ATP at 100 microM and 300 microM induced a reduction in TNF- α secretion, and an increase in IL-10 secretion (a). No effect was seen on IL-6 release. In the presence of 1, 5 and 10 mM H₂O₂, ATP at concentrations of 100 microM and 300 microM still inhibited TNF- α release and stimulated IL-10 release in stimulated blood (b).

Our results demonstrate that even under circumstances of severe oxidative stress, ATP has marked anti-inflammatory properties in stimulated whole blood.

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OXIDATIVE STRESS & INFLAMMATION

January 26, 2007, Pasteur Institute - Paris

Oxidative stress induces exaggerated IL-8 secretion via the activation of ERK1/2 in CF lung epithelial cells

E. Boncoeur, E. Bonvin, C. Muselet-Charlier, A. Clement, MD, J. Jacquot, and TABARY Olivier

Inserm, UMR S 719, Université Pierre et Marie-Curie-Paris6 ; Hôpital Saint-Antoine., Paris, 75012, France

Dysregulated IL-8 production is reported to occur in the lung epithelium of patients with cystic fibrosis (CF). We document here the effects of dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) protein on the production of interleukin (IL)-8 and on the expression of the IL-8 related CXCR1/2 receptors. We examine the implicated signalling pathways in a human bronchial epithelial cell line derived from a CF patient (CF IB3-1, CFTR genotype deltaF508/W1282X) compared with its wtCFTR-corrected cell line (S9 cells) in response to oxidative stress. We show that IL-8 production and expression of CXCR1/2 receptors is increased in both CF IB3-1 and S9 cells under oxidative stress. The p38 and JNK MAP kinase signalling pathways are similarly activated in two CF IB3-1 and S9 cells under oxidative stress. In contrast to that observed in S9 cells, sustained activation of ERK1/2 was unexpectedly revealed in oxidative stress-exposed CF IB3-1 cells and no activation of nuclear factor NF-kappaB was observed. Furthermore, we show that the strongest reduction in both IL-8 production and CXCR1/2 expression is obtained by treatment of oxidative stress-exposed CF IB3-1 cells incubated with the ERK1/2 inhibitor UO126 and for oxidative stress-exposed S9 cells incubated with the NF-kappaB inhibitor BAY 11-7082. All these results were confirmed in bronchial epithelial cell line CFBE41o- (CFTR genotype delta508/delta508) and in the wild type cell line 16HBE14o-.

Our data support the concept that ERK1/2 kinases are key regulators of both IL-8 production and CXCR1/2 receptor expression in CF bronchial epithelial cells when exposed to oxidative stress.

Supported by grants from Inserm, Fondation pour la Recherche Médicale and French cystic fibrosis association (VLM).

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OXIDATIVE STRESS & INFLAMMATION

January 26, 2007, Pasteur Institute - Paris

Differences in caspase involvement in nitric oxide and peroxynitrite-induced cell death in human neutrophils

TAYLOR Emma L., Adriano G. Rossi

Centre for Inflammation Research, University of Edinburgh, Edinburgh, Scotland, UK

The processes of inflammation and apoptosis are linked, and both are regulated by the gaseous free radical, nitric oxide (NO). Apoptosis and subsequent phagocytic clearance of inflammatory cells is necessary for their effective clearance, and consequent resolution of inflammation. Defects in these processes may lead to chronic inflammation and tissue damage caused by persistence of neutrophils, which release a number of pro-inflammatory and histotoxic contents.

We have previously demonstrated that two distinct pure NO donors, diethylamine NONOate (DEA/NO; very fast rate of NO release) and spermine NONOate (SPER/NO; slower release of NO), induce characteristic events of apoptosis in primary human neutrophils at high concentrations (0.31 mM). However, not all apoptotic events correlate with each other, and the time course of apoptosis differs between these compounds, with DEA/NO producing evidence of apoptosis very rapidly, and SPER/NO only inducing apoptosis at later time points (16 hours and onwards), suggesting differing mechanisms.

Here we further investigated the mechanisms of NO-induced neutrophil apoptosis by examining activities of caspase enzymes (effectors of apoptotic cell death), and mitochondrial permeability in neutrophils isolated from the peripheral blood of healthy volunteers. Both total caspase activity and four specific caspases - caspases 2, 3, 8 and 9 - were assessed. We found that DEA/NO activated caspases very early during the incubation period, whereas we could detect no caspase activity above control levels on treatment with SPER/NO at any time point. Analysis of specific enzymes showed concentration-dependent activation of caspases 2 and 3, with no role for caspases 8 or 9, on exposure to DEA/NO. Treatment with SPER/NO led to very limited activation of caspases 2 and 3. Analysis of mitochondrial permeability showed that DEA/NO initiated mitochondrial permeabilisation, despite the lack of caspase 9 activity, whereas SPER/NO-treated neutrophils showed no such mitochondrial effects.

These data suggest that the fast-releasing NO donor, DEA/NO and the slower-releasing compound, SPER/NO, act via different mechanisms to promote apoptosis. Furthermore, whereas DEA/NO acts rapidly to initiate caspase activation (particularly caspases 2 and 3), leading to mitochondrial permeability, SPER/NO-induced apoptosis may proceed via a caspase and mitochondria-independent mechanism.

ABSTRACT 32

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January 26, 2007, Pasteur Institute - Paris

Neutrophils rapidly migrate via lymphatics after Mycobacterium bovis BCG intradermal vaccination and shuttle live bacilli to the draining lymph node

Valérie Abadie¹, Edgar Badell¹, Patrice Douillard², Danielle Ensergueix¹, Pieter J. M. Leenen³, Myriam Tanguy¹, Laurence Fiette¹, Sem Saeland², Brigitte Gicquel¹ and WINTER Nathalie¹

¹Mycobacterial Genetics Unit and the ⁴Laboratory of Histotechnology and Pathology, Institut Pasteur, Paris, France; ²Laboratory for Immunological Research, Schering Plough Dardilly, France; and the ³Department of Immunology, Erasmus Medical Center, Rotterdam, the Netherlands

The early innate response after Mycobacterium bovis BCG vaccination is poorly characterized, but probably decisive for subsequent protective immunity against tuberculosis. Therefore, we vaccinated mice with fluorescent BCG strains in the ear dorsum, as a surrogate of intradermal vaccination in humans. During the first three days, we tracked BCG host cells migrating out of the dermis to the auricular draining lymph nodes (ADLN). Resident skin dendritic cells (DCs) or macrophages did not play a predominant role in early BCG capture and transport to ADLN. The main BCG host cells rapidly recruited both in the dermis and ADLN were neutrophils. Fluorescent green or red BCG strains injected into non-overlapping sites were essentially sheltered by distinct neutrophils in the ADLN capsule indicating that neutrophils had captured bacilli in peripheral tissue and transported them to the lymphoid organ. Strikingly, we observed BCG-infected neutrophils in the lumen of ear lymphatic vessels by confocal microscopy on ear dermis. Fluorescent-labeled neutrophils injected into the ears, accumulated exclusively into the ipsilateral ADLN capsule after BCG vaccination. Thus, we provide in vivo evidence that neutrophils, like DCs or inflammatory monocytes, migrate via afferent lymphatics to lymphoid tissue and can shuttle live microorganisms.

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