

## Publishable executive summary - 2007

### Work performed and results achieved so far

After the third year the partners report good progress, and for all work packages papers have been published. Here follows examples of work and results obtained, for each WP.

**WP2.** Research in this WP concerns the biochemistry of cyclooxygenases, lipoxygenases, LTA<sub>4</sub> hydrolase, and LTC<sub>4</sub> synthases (LTC4S). The structures of two enzymes in eicosanoid biosynthesis was reported. LTC4S converts LTA<sub>4</sub> to the cys-LT LTC<sub>4</sub>. The crystal structure of human LTC4S, in its apo and GSH-complexed forms, reveals a homotrimer, where each monomer is composed of four transmembrane segments. The active site enforces a horseshoe-shaped conformation on GSH, and effectively positions the thiol group for activation by a nearby arginine at the membrane-enzyme interface. This is a remarkable scientific achievement and represents the third crystal structure of a human integral membrane protein ever described. The structure gives important novel insights to the catalytic mechanism of the enzyme, as well as other members of the MAPEG family and is of considerable interest for drug-design.

Phospholipid Hydroperoxide Glutathione Peroxidase-4 regulates cellular redox tone, and thus the activity of COX and LOX. The monomeric structure consists of four  $\alpha$ -helices and seven  $\beta$ -strands. The catalytic triad is localized on a flat impression on the protein surface. The lack of an exposed surface-loop domain may explain the broad substrate specificity of GPx-4. The strong tendency to polymerize was prevented by reductants.

**WP3.** Research in this WP concerns gene regulation of enzymes in the eicosanoid cascade. A novel mode of action of the histone deacetylase inhibitor trichostatin A was described, regarding 5-LOX gene promoter activity. Thus, TSA gave recruitment of Sp1 and Sp3 in living cells (ChIP assay), but not so *in vitro*. Functional VDREs were found, not only in the promoter, but also in several 5-LOX gene introns. A VDRE in intron 4 (42,000 nt downstream of the TSS) is one of the strongest VDREs in the human genome. This gives further support for a role of distally located response elements, regarding control of 5-LOX expression.

**WP4.** Research in this WP concerns roles of eicosanoids in inflammation and immunity.

- Lipoxins (LXs) are endogenously produced anti-inflammatory agents that modulate leukocyte trafficking and stimulate nonphlogistic macrophage phagocytosis of apoptotic neutrophils. Annexin-1 was reported as a factor released from apoptotic cells, stimulating their phagocytosis. The LXA<sub>4</sub> receptor was shown to be coupled to SH2 domain containing tyrosine phosphatase-2 (SHP-2) within a lipid raft microdomain, leading to dephosphorylation of the PDGF receptor  $\beta$ . Lipoxin analogues were synthesized. These analogues will be very useful in studies of LX functions, and are now available for other partners of the consortium.
- Nuclear factor of activated T cells (NFAT) regulates gene transcription (i.e. for COX-2) during the immune response. Mechanisms for regulation of NFATc2 activation have been elucidated.

- A novel effect of LTB<sub>4</sub> in human PMNL, i.e. release of the antibacterial peptide LL-37, was reported. It was shown that LTB<sub>4</sub>, at low (1 nM) concentrations, promotes release of LL-37 peptides from human PMN in a time- and dose-dependent manner. Apparently, in human PMNs, positive feedback circuits exist between LL-37 and LTB<sub>4</sub> that reciprocally stimulate the release of these mediators with the potential for synergistic bioactions and enhanced immune responses.
- Sphingosylphosphorylcholine (SPC) is a bioactive lipid that binds to G protein-coupled-receptors and activates various signaling cascades.
- In renal mesangial cells, SPC not only activates various protein kinase cascades but was also reported to activate Smad proteins, which are classical members of the transforming growth factor- $\beta$  (TGF $\beta$ ) signaling pathway. Consequently, SPC is able to mimic TGF $\beta$ -mediated cell responses, such as an anti-inflammatory and a profibrotic response. Furthermore, the sphingolipid analogue FTY720, was found to have anti-inflammatory activity in mesangial cells.

**WP5.** Research in this WP concerns roles of NO in inflammation and immunity.

- Formation of NO in the GI-tract was further studied. Nitrate is abundant in diet with high levels in many vegetables. Ingested nitrate is reduced to nitrite by bacteria in the oral cavity. It was presented that application of nitrite-containing saliva to the gastric mucosa increases superficial blood flow and mucus generation via acid-catalyzed formation of bioactive nitrogen oxides including NO. Also, dietary supplementation with nitrate would protect against gastric damage caused by a nonsteroidal anti-inflammatory drugs. NO generation in the stomach is greatly enhanced by polyphenols (in red wine) *in vivo* in humans.
- The role of NO in relation to cytochrome c oxidase, and the impact on hypoxic vasodilation. Nitric oxide (NO), generated endogenously in NO-synthase-transfected cells, increases the reduction of mitochondrial cytochrome c oxidase (CcO) at O<sub>2</sub> concentrations ([O<sub>2</sub>]) above those at which it inhibits cell respiration. The two effects of NO is related to electron turnover of the enzyme. The results indicate that partial inhibition of CcO by NO leads to an accumulation of reduced cytochrome c and, consequently, to an increase in electron flux through the enzyme population not inhibited by NO. Thus, respiration is maintained without compromising the bioenergetic status of the cell. This may be a physiological mechanism regulated by the flux of electrons in the mitochondria and by the changing ratio of O<sub>2</sub>:NO, either during hypoxia or, as a consequence of increases in NO, as a result of cell stress.
- Pharmacological manipulation of cytochrome c oxidase indicated that this enzyme, when it is in turnover and in its oxidized state, inactivates physiological amounts of NO, thus regulating its intra- and extracellular concentrations. This inactivation is prevented by blocking cytochrome c oxidase with inhibitors, including NO. Furthermore, when cells generating low concentrations of NO respire toward hypoxia, the redox state of cytochrome c oxidase changed from oxidized to reduced, leading to a decrease in NO inactivation. The resultant increase in NO concentration could explain hypoxic vasodilation.

- The mechanism by which IL-4 downregulates iNOS expression in macrophages in response to inflammatory stimuli, was studied. Taken together, the data suggest that the TNF- $\alpha$  produced in response to IFN- $\gamma$  is required for iNOS induction by activating NF- $\kappa$ B transcription factor.

**WP6.** Research in this WP concerns COX and PG synthases in cardiovascular disease. Retinoid X receptors (RXRs) are transcriptional nuclear hormone receptors, acting as either homodimers or the binding partner for several human nuclear receptors. Functional nongenomic effects of nuclear receptors are poorly understood; however, recently peroxisome proliferator-activated receptor (PPAR) $\gamma$ , PPAR $\beta$ , and the glucocorticoid receptor have all been found active in human platelets. It was proposed that RXR ligands may have beneficial clinical actions through inhibition of platelet activation. The results also demonstrate a novel nongenomic mode for nuclear receptor action and a functional cross-talk between G-protein and nuclear receptor signaling families.

**WP7.** Research in this WP concerns lipoxygenase products in cardiovascular disease. From further studies of transgenic ApoE(-/-) mice, it was reported that a dominant-negative TGF $\beta$  type II receptor conferred upregulation of the FLAP gene, in aorta and adipose tissue. TGF- $\beta$  is a major antiinflammatory mediator in atherosclerosis. Transgenic ApoE(-/-) mice with a dominant-negative TGF $\beta$  type II receptor (dnTGF $\beta$ R2) on CD4(+) and CD8(+) T cells display aggravated atherosclerosis. The aim of the present study was to elucidate the mechanisms involved in this enhanced inflammatory response. Gene array analyses identified FLAP among the most upregulated genes in both the aorta and adipose tissue. The results of the present study suggest a key role for mediators of the 5-LOX pathway in inflammatory reactions of atherosclerosis and metabolic disease.

**WP8.** The research in this WP is focussed on the roles of NO and NO-synthases in vascular physiology and cardiovascular disease. Data were presented:

- On the pharmacological properties of compounds NCX 1512 and NCX 1514, synthesized by linking the histamine H1-receptor antagonist cetirizine to NO-releasing spacer groups. The compounds act as antihistamines and NO donors. However, there was no improved effect compared to cetirizine on antigen-induced constriction of the central and peripheral lung.
- On the farnesoid X receptor/bile acid receptor (FXR; NR1H4), a ligand-activated transcription factor that regulates bile acid and lipid homeostasis. FXR is highly expressed in enterohepatic tissue, but also in vascular tissue. The possibility that FXR regulates inflammation and migration in vascular smooth muscle cells was investigated. The observations suggest that a FXR-SHP pathway may be a novel therapeutic target for vascular inflammation, remodeling, and atherosclerotic plaque stability.

**WP9.** Research in this WP is focussed on the roles of eicosanoids and NO in diseases of the central nervous system. Data were presented on the cerebral function of Phospholipid hydroperoxide glutathione peroxidase (GPx4). Cell-specific enzyme expression at various stages of perinatal brain maturation was investigated, and also its regulation following brain injury. Apparently, astrocytic upregulation of GPx4 in response to injury is part of a protective cascade counteracting further cell damage.

**WP10.** Research in this WP concerns COX, PG synthases, and NO in oncogenesis. In squamous cell carcinoma, the levels of nitric oxide (NO) derived from iNOS and PGE<sub>2</sub> derived from COX-2, produced from tumor cells or tumor-associated inflammatory cells, have been correlated with tumor growth, metastasis, and angiogenesis. The results indicate that iNOS/GC signaling is a downstream player in the control of EP2/EGFR-mediated tumor cell proliferation and invasion.

COX-2, the gastrin-release peptide (GRP) and its cognate receptor (GRP-R) are overexpressed in a significant percentage of colorectal carcinomas and are associated with cell growth, invasiveness and tumor progression. However, a molecular link between these players in adenocarcinomas has not been established. It was found that bombesin (BBS), a GRP homolog, stimulates the expression of COX-2 mRNA and protein in human colon adenocarcinoma Caco-2 cells, resulting in enhanced release of PGE<sub>2</sub>. The findings provide the first evidence for the involvement of the Ca<sup>2+</sup>/Cn/NFAT pathway in BBS-mediated induction of genes involved in colon carcinoma invasiveness such as COX-2.

**WP11.** Research in this WP concerns roles of lipoxygenases in tumor development. Expression of eicosanoid-related enzymes (COX-1, COX-2, 15-LOX-1, 15-LOX-2, 5-LOX) in normal and malignant human ovarian tissue was determined by RT-PCR. A 22-fold elevated expression of 15-lipoxygenase-2 in malignant specimens were found compared to normal ovarian tissue. In ovarian carcinoma metastases, expression of the enzyme was also augmented (20-fold upregulation). The data indicate that 15-LOX-2 may be a candidate tumor marker.

**WP12.** Research in this WP concerns roles of COX, PG synthases and NOS in angiogenesis. The role of the nuclear receptor peroxisome-proliferator activated receptor (PPAR)- $\beta/\delta$  in endothelial cells is unclear. The selective PPAR $\beta/\delta$  ligand GW501516 is in phase II clinical trials for dyslipidemia. The involvement of PPAR $\beta/\delta$  in endothelial cell proliferation and angiogenesis, was studied. Western blot indicated PPAR $\beta/\delta$  is expressed in primary human umbilical and aortic endothelial cells, and in the endothelial cell line, EAHy926. Treatment with GW501516 increased human endothelial cell proliferation and morphogenesis in cultures *in vitro*, endothelial cell outgrowth from murine aortic vessels *in vitro*, and angiogenesis in a murine matrigel plug assay *in vivo*. In conclusion, PPAR $\beta/\delta$  is a novel regulator of endothelial cell proliferation and angiogenesis through VEGF.

**WP 13.** Informations about unique reagents, cell lines and state-of-the-art technologies were gathered from all the member of the Consortium through a questionnaire, and posted through the website of the Consortium in the restricted area. This initiative have prompted collaboration between partners centered around specific technologies developed by participants as well as through the use of unique reagents. Genomic and proteomic basic techniques have also been kept available. Technology for screening novel chemical entities and database of chemical compounds is being made available to the Consortium by Biolipox. The model of isolated and perfused rat brain has been established by UNIMI and will be developed and made available to member of the Consortium interested in evaluating compounds active on cerebral vessels. The *in vivo* pig model is running and available as a core facility.

**WP14.** The partners made an inventory of the educational needs and plan for the corresponding courses and workshops. Educational and training activities at different partner institutions were collected and have been made available at the Consortium level. We have assembled an operative Education and Training Committee and allocated postgraduate courses that cover scientific, technical and management skills. A summer school for graduate students was held in Aigen, Austria, that covered experimental and clinical aspects of the arachidonic acid pathway. Information material, e.g. website and newsletters has been produced and updated.

**WP15.** Our Project Office with Director, Project Managers and Project Administrator now operates to serve the needs of the Consortium. Tools used to facilitate transfer of information and knowledge among the consortium members, have been continuously updated. These include, an intranet site, which allows project members to exchange files, work with a common calendar, have a common archive for files, have discussions and exchange information. In addition, all partners have access to a web based conferencing system. Meetings of the Work Management Group are held regularly every second week using this software.

To get a fruitful collaboration within the project, the Consortium gathers at least once a year for discussions on scientific progress of the project as well as general issues. The annual meeting was held in Aigen in September 2007. The meeting of the Project Steering Committee (PSC) was held in direct conjunction with the annual meeting.

#### **Expected end results and intentions for use and impact**

Eicosanoids and NO are involved in a number of severe endemic diseases addressed in FP6, e.g. atherosclerosis, myocardial infarction, thrombosis, dementia and cancer. In addition, these inflammatory mediators (eicosanoids and NO) are involved in many other disorders. Examples are diseases of the respiratory system (asthma), autoimmune disorders, and the sequelae after severe trauma. Together, these conditions account for the vast majority of mortality and morbidity, in Europe as well as the rest of the world.

As inflammatory mediators, eicosanoids and NO also have beneficial effects in normal physiology. In normal life, we constantly defend ourselves against pathogens, and inflammation is part of normal mechanisms, for example preventing tumorigenesis. Consequently, eicosanoids and NO most probably are of relevance also for diseases characterized by defective defence mechanisms, such as HIV.

Thus, our project is aimed at increasing the knowledge about the mechanisms by which eicosanoids and NO trigger and maintain physiological and pathophysiological processes, in both health and disease. In addition, we aim to identify new drug targets, evaluate the therapeutic potential of recently developed lead structures, improve existing therapies and develop novel drugs and therapeutic strategies. Hence, our project has the potential to prolong the life expectancy and significantly improve the health, quality of life, and prosperity of the citizens of Europe.

### Main elements of the plan for using and disseminating knowledge

During the third year of the Eicosanox project 62 articles, with an average impact factor of 6.0 have been published in scientific journals, and the results have been presented at several international conferences. To disseminate information to other scientists and to the public, the external website has been updated in a regular manner (<http://www.eicosanox.org/>).

## Eicosanox publications

62 published articles during 2007

Average impact factor 6.0

