Oxidized LDL induces the production of TNF-alpha in human THP-1 cells.

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Atherosclerosis is a chronic inflammatory process where oxidative damage within the artery wall is implicated in the pathogenesis of the disease. It is now well accepted that oxidative modification of low-density lipoprotein (LDL) contributes to the pathology of atherosclerosis. Myeloperoxidase (MPO), an enzyme secreted by activated phagocytes is considered to be responsible for LDL oxidation, in the intima, converting the LDL into a high-uptake form for macrophages. In this study we aim to show that MPO-oxidized-LDL are specifically involved in the initiation of an ongoing inflammatory response that is mediating all stages of atherosclerosis. To do so, albumin, the most representative plasma protein that can also be oxidized within the circulation was chosen to see the effect of its oxidative form on triggering an inflammatory response.

Lipoproteins were isolated from plasma by use of density-gradient ultracentrifugation (d = 1.019-1.063). LDL and albumin were gel filtered (PD-10, Pharmacia) and 1.0 mg of protein was oxidized by 2.6 chlorinating units of recombinant MPO, to form the fraction of Mox-LDL and Mox-albumin in the presence of 1 mM H₂O₂ in PBS for 5 minutes at 37°C. A native 7.5 % polyacrylamide gel electrophoresis was used to check out the oxidation of albumin. Using monoclonal antibodies directed against Mox-LDL, oxidized albumin were not recognized showing the specificity of these antibodies for Mox-LDL.

Human THP-1 cells (human monocyte cell line) were incubated for 4 hours at 37°C under 5% CO₂, in the presence or absence of 100μg/ml of Mox-albumin or Mox-LDL. The production of TNF-alpha was in the supernatant measured after 4 hours by an Elisa assay (Roche). Our results showed that Mox-LDL (2 ± 0.2) induced a 2-fold increase in the TNF-alpha production compared to native LDL (1.2 ± 0.28) and cells alone. On the other hand, native albumin (0.6 ± 0.1) decreased this production while the Mox-albumin (1 ± 0.23) does not produce TNF-alpha. As a positive control we used LPS (4.9 ± 0.19) known to produce a high amount of TNF-alpha.

In conclusion, we may propose that the oxidized LDL in the circulation in opposition to native LDL, are able to trigger an inflammatory process that is known to promote initiation and evolution of atherosclerosis. And the albumin has no apparent role in this initiation.

References
Human peroxiredoxin 5 protects nuclear and mitochondrial DNA against oxidative attacks induced by peroxides

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Peroxiredoxins (PRDXs) are a family of peroxidases well conserved from bacteria to humans. Six members have been identified in mammals. Mammalian PRDXs reduce peroxides with the use of reducing equivalents derived from thioredoxin, glutathione or cyclophilin A. Amongst those six members, PRDX5 has the widest subcellular localization. Indeed, it can be addressed intracellularly to mitochondria, peroxisomes, the cytosol and, to a lesser extent, the nucleus. Our previous work has shown that overexpression of human PRDX5 in different subcellular compartments of CHO cells significantly reduced cell death (BANMEYER et al., 2004). The present work focused on the genoprotective capabilities of PRDX5 in mitochondria and in the nucleus. First, we investigated whether PRDX5 can directly protect DNA from damages induced by hydroxyl radical by studying its effects on DNA strand breaks generated by metal-catalyzed oxidation with dithiothreitol as electron donor (MCO-DTT). We assessed DNA strand breaks by measuring the conversion of plasmid DNA from its intact form (supercoiled DNA) to its damaged form (open circular). Our results showed that recombinant PRDX5 partially prevented DNA strand breaks by the MCO-DTT system, with complete DNA protection at concentrations of 40 μg/ml. Second, we investigated the genoprotective effect of PRDX5 on mitochondrial DNA (mtDNA). MtDNA has been reported to be more susceptible to ROS-induced damage than nuclear DNA because of the lack of histones and because of the absence of effective system for repairing oxidative DNA lesions in mitochondria. We assessed mitochondrial DNA damages by long PCR experiments. The PCR method is based on the fact that DNA damages, such as strand breaks and abasic sites, can hamper the progress of the polymerase. As a result, a reduction in the relative yield of mitochondrial long PCR product reflects the presence of blocking DNA lesions. DNA damages were induced by exogenously added hydrogen peroxide or tert-butylhydroperoxide. Our results showed that mitochondrial PRDX5 (overexpressed up to 3.4-fold) can confer an important protection to mtDNA in presence of hydrogen peroxide, but no significant protection in presence of tert-butylhydroperoxide. Third, we investigated the genoprotective effects of PRDX5 on nuclear DNA (nDNA), in presence of exogenously added hydrogen peroxide or tert-butylhydroperoxide. We measured nDNA damages using the comet assay, which detects strand breaks and abasic sites at the cell level. Overexpression of PRDX5 in CHO cells up to 6-fold in the nucleus conferred a significant protection against nDNA damages induced by both oxidants. In conclusion, PRDX5 can protect DNA in vitro against oxidative attacks induced by hydroxyl radical. In CHO cells, high levels of mitochondrial PRDX5 can protect mitochondrial DNA against oxidative attacks caused by peroxides. PRDX5 can also act as an alkyl hydroperoxide reductase in the nucleus, suggesting a genoprotective role for nuclear PRDX5.

This work was financially supported by the FNRS and by the « Communauté française de Belgique-Actions de Recherche Concertées ». I. Banmeyer is recipient of a FRIA fellowship.

References
Multiple signalling pathways initiated by oxidative stress regulate cancer cell death in response to Photodynamic Therapy

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Photodynamic Therapy (PDT) is an approved treatment for different types of tumors and certain benign diseases, which is based on the use of a light-absorbing compound (photosensitizer) and local light irradiation (Dolmans et al., 2003). Light-activation of the photosensitizer accumulated in cancer tissues in the presence of oxygen leads to the local production of reactive oxygen species (ROS) that kill the tumor cells either by apoptosis or necrosis. PDT-induced apoptosis is mediated mainly by a mitochondria-regulated pathway, which is inhibited by the overexpression of the caspase-9 and –3 inhibitor XIAP but not by the viral caspase-8 inhibitor CrmA (Assefa et al., 1999). The photogeneration of ROS triggers the mobilization of the pro-apoptotic Bax protein from the cytoplasm to the mitochondria, leading to the permeabilization of the mitochondrial outer membrane, release of cytochrome c into the cytosol and activation of the effector caspase-3. However, PDT also causes the activation of signalling pathways involved in adaptive responses that may enable the cancer cells to repair the damage and to survive. Signaling pathways regulated by members of the Mitogen Activated Protein Kinases and their downstream targets, such as cyclooxygenase-2, appear to critically modulate cancer cell sensitivity to PDT (Hendrickx et al., 2003). Understanding the molecular events that contribute to PDT-induced apoptosis and how cancer cells can evade apoptotic death, should enable a more rationale approach to drug design and therapy.

This work was supported by the Geconcerteerde Onderzoeksactie (GOA, from the KULeuven) and by the Interuniversitaire Attractiepolen (IAP, V/P12) of the Federal Belgian Government.

References
Low glucose-induced apoptosis of insulin-producing MIN6 cells involves production of reactive oxygen species

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Prolonged exposure to low glucose levels induces apoptosis in mouse insulin-producing MIN6 cells as well as in rat primary beta cells [Hoorens et al., 1996; Kefas et al., 2003]. In the accompanying study (by Martens G. et al.), we demonstrate that acute reduction rather than acute increase of glucose metabolism stimulates the mitochondrial ROS production in purified rat beta cells, which in turn increases their apoptosis rate. These findings are somewhat at variance with recently reported data in MIN6 cells suggesting that glucose increases the intracellular ROS derived from the mitochondrial electron transport chain [Sakai et al., 2003]. Therefore, we re-examined how the production of reactive oxygen species (ROS) in MIN6 cells is influenced by prolonged incubation (upto 24 h) in a low glucose concentration, and whether this could be relevant to their apoptosis in that condition.

The mean oxidation-dependent fluorescence of the ROS-probe dihydro-dichloro-fluorescein-diacetate (H$_2$-DCFDA), which is also intracellularly de-esterified to DCF, was increased 1.4 and 1.7-fold in MIN6 cells cultured for 6 and 24 h in 0.6 mM glucose (respectively), as compared to MIN6 cells cultured in 25 mM glucose (p<0.01). These results suggested a prolonged increase of ROS-production in low glucose concentrations. In accordance with this, the mRNA expression of heme oxygenase-1 (HO-1), glutathione peroxidase, and catalase, was significantly increased at 15 and 30 h of culture in low glucose. HO-1 upregulation in low glucose was confirmed at the protein level. The ROS-scavengers vitamin E (5 µM, 25 µM), N-acetyl-cysteine (1 mM, 5 mM), and the superoxide dismutase mimetic compound MnTBAP (10 µM), lowered both the ROS-accumulation (DCF-fluorescence) and the apoptosis in MIN6 cells cultured in low glucose. Our results suggest that ROS production plays a role in the apoptosis of MIN6 cells that is induced by their culture in low glucose concentrations. We also found that alternative carbon sources, like pyruvate (5-20 mM), could substitute for glucose in suppressing the ROS production and apoptosis of MIN6 cells.

References
Improvement by solubilization in DMPC liposomes of PPME photodynamic effect – A study in human colon cancer cells HCT-116.

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In view of its potential use in photodynamic therapy (PDT), pyropheophorbide-a methyl ester (PPME) is an attractive second generation photosensitizer derived from chlorophyll a.

Matroule and al. demonstrated that PPME solubilized in medium culture was mainly localized in the endoplasmic reticulum (ER), the Golgi apparatus (GA) and lysosomes of HCT-116 cells. Thus, the active cellular site for triggering apoptosis mediated by PPME photosensitization was the ER-AG system. Apoptosis was due to production while necrosis was due to the production of singlet oxygen.

It has been shown before that liposomes enhance the photodynamic activity of active drugs. This effect is related to the monomerization of most photosensitizers in liposomes. Consequently, we have solubilized PPME in aqueous dispersion of small unilamellar DMPC vesicles. In this work, incubation of HCT-116 cells with PPME in liposomes followed by irradiation with red light increases cellular death as indicated by the Trypan blue exclusion method. Cells in presence of PPME solubilized previously in DMPC liposomes were more fluorescent. FACS analysis of PPME stained with Annexin V/PI was used to compare the ratio between apoptosis and necrosis. In presence of liposomes, PPME induced a more important apoptosis than in the case of alone PPME. Using confocal laser scanning microscopy and organelle specific fluorescent probes, PPME was found to localize in the intracellular membrane system, namely the endoplasmic reticulum, the Golgi apparatus, lysosomes and mitochondria.

To conclude, PPME incorporated into liposomes improves the cytotoxic effect of the PDT on HCT-116 cells line by a change in localization and an increase of apoptosis.
Soy phytoestrogens counteract MSK1-dependent NF-κB transactivation, partly by their anti-oxidant activity

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After menopause or andropause, loss of the normally inhibiting sex steroids (estrogen, testosterone) results in elevated IL6 levels, which are further progressively increasing with age. This aberrant gene expression accounts for several disease-associated pathologies and phenotypical changes of advanced age, such as osteoporosis, rheumatoid arthritis, multiple myeloma, neurodegenerative diseases, frailty (VANDEN BERGHE et al., 2000). Excessive IL6 expression also promotes tumorigenesis (breast, prostate, lung, colon, ovarian) and serum IL6 levels are currently regarded as a diagnostic marker for tumor progression and prognosis in various cancers. In order to prevent these types of complaints and to maintain the important physiological balance, estrogens and SERMs (such as raloxifene, tamoxifen) have long been recommended in hormone replacement therapy (HRT). Unfortunately, both pharmacological compound groups have been associated with severe negative side effects. In this view, plant-derived estrogens (phytoestrogens), particularly those found in soy products (such as genistein), are considered as putative beneficial alternatives in HRT. We have analysed in more detail how soy isoflavones (genistein, daidzein, biochanin A) inhibit expression of the ‘geriatric’ cytokine IL6. TNF-induced IL6 production in fibroblasts mainly depends on nuclear translocation of the transcription factor NF-κB, as well as on the activation of p38 and p42/p44 MAPKs, and consequent cofactor recruitment to the IL6 promoter (VANDEN BERGHE et al., 1998; 1999). Recently, our research unit identified the dual MAPK (p38, ERK)-responsive MSK1, as an important player for nuclear NF-κB phosphorylation (transactivation), besides chromatin components (i.e. H3), for optimal IL6 gene transcription (VERMEULEN et al., 2002; 2003). With respect to molecular targets of soy isoflavones, our results demonstrate that soy phytoestrogens, but not synthetic 17β-estradiol, can counteract MSK-dependent NF-κB transactivation on selective target genes in fibroblasts, most probably via its tyrosine kinase inhibitor and anti-oxidant properties. As soy isoflavones integrate both hormonal ligand activities and interference with signaling cascades, therapeutic use may not be restricted to hormonal ailments only, but may have applications in cancer chemoprevention and/or NF-κB-related inflammatory disorders as well.

References

Human peroxiredoxin 5 is a peroxynitrite reductase

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Peroxiredoxins (PRDXs) are a ubiquitous family of peroxidases widely distributed among prokaryotes and eukaryotes. PRDX5, which is the last discovered mammalian member, was previously shown to reduce peroxides with the use of reducing equivalents derived from thioredoxin. Loss of electrons along the mitochondrial electron-transfer chain produces superoxide, that reacts rapidly with nitrogen monoxide, and produces peroxynitrite. The latter is toxic to the cell and is considered to be the main culprit in the pathological actions of nitrogen monoxide. Recently, alkyl hydroperoxide reductase subunit C (AhpC), a bacterial PRDX homologue of human PRDX5, was reported to be able to reduce peroxynitrite to nitrite, which demonstrates the peroxynitrite reductase activity of prokaryotic peroxiredoxins (Bryk et al., 2000). In the view of this, we hypothesized that human PRDX5, could also exhibit peroxynitrite reductase activity and protection against peroxynitrite. We report here that recombinant human PRDX5 protects dihydrorhodamine 123 from oxidation caused by peroxynitrite. Analysis of mutant PRDX5 in which each of the cysteine residue implicated into peroxide reduction was mutated suggests that the nucleophilic attack on the O-O bond of peroxynitrite is performed by the N-terminal peroxidatic Cys47. Moreover, the oxidation of Cys47 by peroxynitrite was reversible in the presence of the thioredoxin system. Also, quantification of nitrite and nitrate by ion chromatography indicated that PRDX5 successfully competed with the spontaneous decay of peroxynitrite into nitrate. Indeed, an increase in nitrite was measured in the presence of human PRDX5, confirming enzymatic peroxynitrite reduction into nitrite.

With the use of pulse radiolysis, we show also that human recombinant PRDX5 reduces peroxynitrite with an unequalled high rate constant of \((7 \pm 3) \times 10^7 \text{ M}^{-1}\text{s}^{-1}\). This rate constant is 50 times higher than that reported for its bacterial homologue and 10 times higher that of mammalian glutathione peroxidases. Considering these results and the preferential mitochondrial localisation of PRDX5, we performed in vitro work indicating that PRDX5 is a highly efficient peroxynitrite reductase that may play a major protective role against mitochondrial peroxynitrite toxicity in mammalian cells.

This work was financially supported by ETHZ (Zürich) and by grants from the « Communauté française de Belgique-Action de Recherches Concertées » and from the « Fonds pour la Formation à la Recherche dans l’Industrie et l’Agriculture ».

References
Regulation of human peroxiredoxin 5 expression and identification of phosphorylation sites

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Peroxiredoxins (PRDXs) are a family of peroxidases expressed at high levels and widely distributed in eukaryotes and prokaryotes (Wood et al., 2003). PRDXs reduce peroxides including hydrogen peroxide and alkyl hydroperoxides, but also peroxynitrite. PRDX5, the last discovered member of mammalian PRDXs, is targeted to mitochondria, peroxisomes and the cytosol. PRDX5 protects mammalian cells from oxidative attacks by reactive oxygen species (ROS) and reactive nitrogen species (RNS) in pathophysiological situations (Banmeyer et al., 2004).

Although hydrogen peroxide causes oxidative cell damages at high concentrations, it may also serve as an intracellular messenger. Due to their peroxidase activities, PRDXs appear to be able to modulate hydrogen peroxide-dependent signal transduction pathways in addition to their antioxidant protective role.

The main objective of this study is to determine whether PRDX5 expression or its enzymatic activities might be regulated in response to cell stimulations and whether these regulations might modulate intracellular pathways.

First, regulation of PRDX5 expression was examined in SH-SY5Y neuroblastoma cell line exposed to oxidative or nitrooxidative molecules such as hydrogen peroxide, SIN-1 (peroxynitrite generator) and pro-inflammatory cytokines able to generate ROS in mammalian cells. Using Western blotting, we showed that PRDX5 expression is not regulated in SH-SY5Y cells by hydrogen peroxide, SIN-1 or three cytokines (IL-1β, TNF-α and IFN-γ). Consequently, we hypothesized that PRDX5 might be submitted to post-translational modifications such as phosphorylation. Using in vitro kinase assays, we showed that human PRDX5 is phosphorylated by cyclic GMP-dependent protein kinase (PKG). We also demonstrated, using Western blotting, that PRDX5 immunoprecipitated from SH-SY5Y cell lysates is phosphorylated on a serine when SH-SY5Y cells are exposed to 10 μM membrane-permeable cGMP analog, 8-pCPT-cGMP, an activator of PKG.

In conclusion, our data show that human PRDX5 expression is not regulated in SH-SY5Y cells exposed to hydrogen peroxide, SIN-1, IL-1β, TNF-α and IFN-γ. However, human PRDX5 appears to be phosphorylated by PKG. This suggests that post-translational modification might regulate peroxidase activities of human PRDX5 and would be able to modulate redox-dependent intracellular signalling pathways.

This work was financially supported by grants from the “Communauté française de Belgique-Action de Recherches Concertées” and from the FNRS. F. Etienne is recipient of a FRIA fellowship.

References
Mechanisms of NF-κB activation by oxidative stress

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Since the discovery that NF-κB could be activated by hydrogen peroxide [1], several laboratories put a lot of efforts into dissecting the molecular mechanisms underlying this activation, particularly in T lymphocytes. These works led to the observation that the mechanism of NF-κB activation by H₂O₂ (including the tyrosine phosphatase inhibitor pervanadate) involved tyrosine phosphorylation of the inhibitor IkBα rather than phosphorylation of serine 32 and 36. The IkB kinase (IKK) complex did not seem to be implicated in this activation [2,3,4].

In the present work, we investigated with more details the possible role of the IKK complex in NF-κB redox regulation. By using three different T cell lines (CEM, Jurkat and Jurkat JR), we clearly showed that pervanadate treatment induces tyrosine phosphorylation of IkBα without activation of the IKK complex in all T cell lines. However, the mechanism of NF-κB activation by H₂O₂ was clearly cell-type dependent: it induced a strong activation of the IKK complex leading to phosphorylation of serine 32 and 36 of IkBα in CEM and Jurkat JR cells, but not in Jurkat cells, where H₂O₂ activated NF-κB through tyrosine phosphorylation of IkBα. These results showed for the first time that NF-κB activation by H₂O₂ could induce a classical mechanism involving IKK complex activation. To further investigate the role of the IKK complex in tyrosine phosphorylation-dependent activation of NF-κB, we used NEMO/IKKγ deficient Jurkat cells. Interestingly, we showed that treatment of NEMO⁻/⁻ Jurkat cells with pervanadate completely abrogated NF-κB activation, as detected by EMSA, but still induced tyrosine phosphorylation and degradation of IkBα, confirming that the IKK complex was not implicated in IkBα tyrosine-phosphorylation, but that NEMO might have a role in the nuclear translocation of NF-κB. To further confirm this hypothesis, we used MEF lacking IKKα and IKKβ. Pervanadate treatment of these cells still revealed a normal tyrosine phosphorylation of IkBα followed by degradation, but no nuclear translocation of NF-κB, suggesting that NEMO require IKKα and/or β for the nuclear translocation of NF-κB. Using IKKα⁻/⁻ and IKKβ⁻/⁻ single knocked-out MEFs, we showed finally that IKKα and NEMO/IKKγ are both required for a correct nuclear translocation of NF-κB after pervanadate treatment. Overall, our results demonstrate the existence of two different mechanisms leading to NF-κB activation after an oxidative stress depending on the cell-type: a classical pathway involving IKK complex activation and phosphorylation of IkBα on serines 32 and 36, and an alternative pathway leading to tyrosine phosphorylation of IkBα without IKK activation. This alternative pathway gave us an interesting tool to evaluate the role of IKK complex subunits in NF-κB activation, independently of their kinase activity. In this way, we showed that both NEMO/IKKγ and IKKα are crucial for NF-κB nuclear translocation.

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Implication of the essential cyclin dependant kinase complex Cdk9/Pch1 in pre-mRNA processing.

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At the contrary of the «cell-cycle» Cyclin-dependent kinases (Cdks), the «transcriptional» cyclin-dependant kinases are involved in the regulation of transcription by phosphorylation of the carboxyterminal domain (CTD) of the RNA polymerase II.

In *Shizosaccharomyces pombe*, we are studying a Cdk/Cyclin complex, Cdk9/Pch1. This complex, described as the ortholog of the human complex P-TEFb, phosphorylates *in vitro* the polII CTD and therefore is suspected to play a role in transcription (Pei *et al.*, 2003).

To elucidate the *in vivo* function of the Cdk9/Pch1 and investigate the network of regulations in which these two proteins are involved, we have purified the complex of proteins associated with them using the TAP method (Tandem Affinity Purification, (RIGAUT *et al.*, 1999)). Using mass spectrometry (MS), we show that Cdk9-Pch1 co-purifies with a protein required for mRNA capping (a pre-mRNA modification coupled with transcription), thereby suggesting a role for Cdk9-Pch1 in mRNA capping.

Since the genes encoding Cdk9 and Pch1 are essential for growth (FURNARI *et al.*, 1997), the next step will be the generation of conditional mutants to study the implication of Cdk9/Pch1 in the regulation of transcription and mRNA capping.

A. G. and L. T. are recipients of a FRIA fellowship, DH is supported by the Human Frontier Science Program (Long term fellowship LT00665/2003). This work is supported by FNRS (convention FRFC 2.4504.00-2003).

References


Genetic profile and physiological role of alternatively activated macrophages in various pathologies

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Depending on the cytokine environment, macrophages can differentiate in distinct subsets, performing specific immunological roles. In this regard, functions of macrophages activated by IFN-γ, referred to as classically activated macrophages (caMF), have been extensively documented. Recently, it has been recognised that macrophages exposed to IL-4 and IL-13 undergo an alternative activation program. However, the nature and functions of alternatively activated macrophages (aaMF) are ill defined. Our aim is to better characterize the genetic/molecular profile, physiological implication and regulation of aaMF elicited during various diseases including African trypanosome and helminth infections, as well as during allergy and cancer.

Suppression subtractive hybridization (SSH) was used to identify genes that are differentially expressed in \textit{in vivo}-elicited caMF versus aaMF, obtained via an experimental murine model of resistance to African trypanosomosis characterized by the development of caMF in the early stage of infection, whereas in the late and chronic stages of infection aaMF develop. Via this approach, we have identified more than 45 genes with distinct expression patterns in aaMF. Besides the earlier documented markers (mannose receptor, arginase, FIZZ1, YM), MGL1 and MGL2, belonging to the C type lectin family, were differentially induced in diverse populations of aaMF elicited during infection with Trypanosoma sp and Taenia crassiceps, in allergic lungs and in mice with progressing tumors. Moreover, MGL1/2 expression was induced by IL-4 in human monocytes. In parallel, by comparing gene expression profile in Trypanosoma resistant and susceptible mice, we found that genes regulating the arachidonic acid metabolism and genes involved in the regulation of the redox status of the macrophages may play a cardinal role in resistance to parasitic disease.

Our work provides novel markers for both murine and human aaMF elicited in various pathological conditions. The genes that were identified provide a molecular basis to investigate a number of documented or suspected properties of alternatively activated macrophages, including immunomodulation, wound healing and anti-inflammation.
Superoxide dismutase mimetics elevate sod activity in vivo but do not retard aging in the nematode Caenorhabditis elegans

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The oxidative damage theory of aging states that the accrual of molecular damage from reactive oxygen species (ROS), particularly superoxide and its derivatives, is the primary cause of aging. EUK-8 and EUK-134, synthetic mimetics of the antioxidant enzyme superoxide dismutase (SOD), are excellent tools to test this theory. In contrast to a previous report (Melov et al., 2000), we could not detect any extension of lifespan (Keaney and Gems, 2003; own observation) upon administration of these mimetics. Similar conclusions were drawn using other animal models such as the housefly (Bayne & Sohal, 2002). We further examined the effect of EUK-8 and EUK-134 in C. elegans both in vitro and in vivo.

Using our high-throughput SOD assay (Lenaerts et al., 2002), we tested the in vivo SOD activity of EUK-8 and EUK-134. This was done with or without EDTA in the assay mixture in order to discriminate between endogenous SOD (not inhibited by EDTA) and SOD activity by EUK only (inhibited by EDTA). We found that treatment with SOD mimetics significantly elevated total SOD activity levels in C. elegans, without altering protein SOD activity. This effect was most obvious in the mitochondria, where up to 5-fold increases were recorded. Reduction of lifespan by the superoxide generator paraquat was counteracted by EUK-8 in a dose-dependent fashion. Yet in the absence of a superoxide generator, treatment with EUK-8 or EUK-134 did not increase lifespan, even at doses that were optimal for pro-oxidant protection. Thus, an elevation of SOD activity levels does not retard aging in the absence of superoxide generators. This suggests that C. elegans lifespan might not be limited directly by levels of superoxide and its derivatives.

We thank B.P. Braeckman, K. Houthooft, T. Magwere, J.J. McElwee, L. Partridge, M.D.W. Piper, P.W. Piper and M. West for discussions and advice. M.K. and D.G. were supported by the Biotechnology and Biological Sciences Research Council (UK), the European Union, the Royal Society, and the Wellcome Trust. F.M. and J.R.V. by Ghent University, the Fund for Scientific Research-Flanders, and M.S. by the Worshipful Company of Pewterers (City of London) and the Brain Research Trust.

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Mammalian peroxiredoxins: protective antioxidant enzymes or regulators of signal transduction?

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Peroxiredoxins (PRDXs) have been identified a decade ago as an ubiquitous family of peroxidases widely distributed among organisms from bacteria to plants and animals. PRDXs are heme-free and selenium-free peroxidases, and compose at least 0.1 to 0.8 % of total soluble proteins of mammalian tissues. These enzymes use redox-active cysteines to reduce hydrogen peroxide, alkyl hydroperoxides but also peroxynitrite. PRDXs are recycled by reducing equivalents derived from thiol-containing donor molecules such as glutathione, glutaredoxin, tryparedoxin, cyclophilin and thioredoxin (WOOD et al., 2003a). In unicellular organisms such as bacteria, yeast and protozoan parasites, PRDXs have been mainly viewed as antioxidant enzymes dedicated to protect cells against reactive oxygen species (ROS) or reactive nitrogen species (RNS). Indeed, their catalytic efficiencies with hydrogen peroxides and peroxynitrite but also their resistance to inhibitory overoxidation of their catalytic cysteines, suggest that their main function is to protect cells from oxidative or nitrooxidative stresses (WOOD et al., 2003b). In multicellular organisms, PRDXs have been implicated in redox-dependent regulation of signal transduction. When overexpressed in various mammalian cell types, PRDX enzymes efficiently reduce the increase in the intracellular concentration of hydrogen peroxide induced by platelet-derived growth factor or tumor necrosis factor-alpha, inhibit the activation of NF-kB induced by tumor necrosis factor-alpha, and block apoptosis induced by ceramide, suggesting that they function in signaling cascades by removing hydrogen peroxide (RHEE et al., 2002). In mammals, six different PRDX genes have been cloned. PRDX5 is the last discovered member (KNOOPS et al., 1999). Interestingly PRDX5 protein can be intracellularly localized to mitochondria, peroxisomes, the cytosol and, to a lesser extent, the nucleus. This remarkably wide subcellular distribution compared with the five other mammalian PRDXs and its localization in organelles that are sources of ROS or RNS, prompted us to further investigate the antioxidant protective function of PRDX5 in mammalian cells according to its subcellular targeting (BANMEYER et al., 2004). Chinese hamster ovary (CHO) cells overexpressing human PRDX5 in the cytosol, in mitochondria, or in the nucleus were established by stable transfection. Cells were exposed to oxidative or nitrooxidative stresses with peroxides, peroxynitrite or nitric oxide. Cell protection conferred by PRDX5 was evaluated by clonogenicity, lactate dehydrogenase or MTT assays. Overexpressing PRDX5 in either the cytosolic, mitochondrial, or nuclear compartment significantly reduced cell death induced by peroxides. Overexpression of mitochondrial PRDX5 afforded also a significant protection against nitrooxidative stress induced by peroxynitrite and nitric oxide. Moreover, partial inactivation of endogenous mitochondrial PRDX5 expression by RNA interference sensitized CHO cells to peroxynitrite and nitric oxide. In conclusion, more attention was focused recently on PRDX roles in mammals as regulators of redox-sensitive signaling. However, the specific subcellular localization of certain PRDXs in organelles that are sources of ROS and RNS, their abundance in many different cell types in mammals and findings that specific inhibition or overexpression of individual PRDXs modulate cell death sensitivity to oxidative and nitrooxidative stresses argue that mammalian PRDXs are also important protective antioxidant enzymes.
This work was financially supported by grants from the “Communauté française de Belgique-Action de Recherches Concertées” and from the FNRS.

References
Putative in vitro antioxidant effect of organometallic analogs of tamoxifen

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Tamoxifen (TAM) and its congeners are widely used as a supplementary therapy to control cancers of the breast that test positive for the oestradiol receptor (FEUER et al, 1993). Despite the increasing survival rate of patients, there is a development of resistance in the long run treatment with TAM. Moreover, TAM was found to stimulate certain tumours of the breast (MAC CREGOR and JORDAN, 1998). Consequently, the design and synthesis of new TAM analogs were undertaken, and the ferrocene derivatives have shown to possess the unusual properties of combining both antiproliferative effects on oestrogen receptor positive breast cancer cell lines and genotoxic effects due to the attached organometallic moiety (TOP et al, 2001).

In this report, we investigated the in vitro antioxidant effect of two analogs of TAM [1-(4-hydroxyphenyl)-1-(phenyl)-2-ferrocenyl-but-1-ene (F1) and 1-di-(4-hydroxyphenyl)-2-ferrocenyl-but-1-ene (F2)] versus TAM, on reactive nitrogen and oxygen species (RNOS) generated by various means: peroxynitrite; MPO/H2O2/NO2- for HOCI and ·NO2; HRP/H2O2/ABTS for ABTS radical cation; Fenton reaction for ·OH radicals and xanthine/xanthine oxidase for superoxide anions. Radical scavenging activity and changes of spectral absorbance were monitored using UV-visible spectroscopy and EPR technique.

Our UV-visible results show that peroxynitrite induces spectral changes with appearance of new peak at 360 and 560 nm respectively for F1 and F2, while no change was observed with TAM. Similar results are obtained with the MPO/H2O2/NO2- system except that the observed peak for F2 is shifted at 420 nm. We also observe the ABTS radical cation formation at 730 nm which is inhibited by F2 and F1, in a concentration-dependent manner. Like in the previous systems, TAM has no significant effect on the radical cation generation. By EPR, F1 and F2 are found only moderate effect on both hydroxyl and superoxide radicals in comparison with controls. TAM has weak radical scavenging effect. On the other hand, with the HRP/H2O2/ABTS system F1 and F2 exhibit a marked inhibiting effect on the EPR signal versus TAM.

In conclusion, EPR and UV-visible results indicate that both ferrocene derivatives (F1 and F2) exhibit an antioxidant activity towards RNOS, especially free radicals. Additional investigation on cell models is currently in progress

References
Single strand breaks and 8-oxodG in sickle cell disease treated by hydroxyurea

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Objectives
This pilot study aims to determine the DNA oxidation status (single strand breaks and 8-oxodG in DNA lymphocytes, 8-oxodG urinary excretion) of patients with sickle cell disease (SCD) and to assess the effect of hydroxyurea treatment on DNA oxidation.

Methods
The Comet assay method was associated with formamidopyrimidine glycosylase (FPG) to assess the DNA single strand breaks and 8-oxodG in lymphocytes; a 8-oxodG E.L.I.S.A test was used to measure the urinary excretion of this oxidized base. We performed the three assays on three groups of children: 7 patients SCD without hydroxyurea, 7 patients with SCD treated by hydroxyurea and 5 patients without SCD as controls.

Results
The Tail DNA (median) for the three groups was between 5 and 30 %. The 8-oxodG excretion ranged between 0 and 3.0 µg /g creatinine.

Conclusions
This pilot study about the DNA oxidation status in sickle cell disease treated by hydroxyurea did not demonstrate a sickle cell disease effect nor an hydroxyurea effect on DNA lymphocytes or 8-oxodG excretion. This study tends to show that long-term hydroxyurea treatment should be reasonably safe in terms of carcinogenicity by DNA oxidative damage.
Protection against nitrooxidative stress conferred by the mitochondrial thioredoxin system in mammalian cells

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Oxidative and nitrosative stresses induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been implicated in the pathophysiology of numerous neurological diseases (ISCHIROPOULOS et al., 2003). Mitochondria are a main source of ROS and RNS in mammalian cells. In this work, we focused on an enzymatic antioxidant system localized in mitochondria, the mitochondrial thioredoxin system (MTS). This system is composed of thioredoxin reductase 2 (TrxR2), thioredoxin 2 (Trx2), peroxiredoxin 3 (PRDX3) and peroxiredoxin 5 (PRDX5). Via electron transfer from NADPH, the MTS is able to reduce hydrogen peroxide, alkyl hydroperoxides but also peroxynitrite (MIRANDA-VIZUETE et al., 2000).

On one hand, we examined the expression of each protein of the MTS in rat central nervous system (CNS). A systematic histochemical analysis was performed. The MTS is mainly expressed in neurons and even highly expressed in neuronal populations such as Purkinje cells, locus coeruleus and mitral cells of olfactory bulb. Furthermore, we focused our attention on certain neuronal populations such as hippocampal neurons and dopaminergic neurons of the substantia nigra (SN) degenerating respectively during Alzheimer’s disease and Parkinson’s disease. In rat hippocampus, the MTS is well expressed in CA2/3, CA4 layers and in the subiculum but weakly expressed in CA1 and gyrus dentatus layers. MTS expression is totally absent in dopaminergic neurons of rat SN.

On the other hand, we evaluated survival of CHO cells overexpressing each protein of the MTS when nitrooxidative stresses are induced by NO and ONOO⁻ donors such as SNAP and SIN-1. Nitrotyrosines, used as markers of nitrosative stress, were released by cells with increasing concentrations of SIN-1 only. When clones overexpressing each protein were exposed to SIN-1 and SNAP, a significant protection was observed in cells overexpressing mitochondrial PRDX5. Moreover, partial inactivation of endogenous mitochondrial PRDX5 expression by RNA interference sensitized CHO cells to SNAP and SIN-1.

Altogether, these data provide evidence that in the MTS, PRDX5 confers a significant protection against nitrooxidative stress. Moreover, our results suggest also that the vulnerability of hippocampal neurons and dopaminergic neurons of the SN could be partly due to a low expression level of mitochondrial PRDX5.

This work was financially supported by grants from the “Communauté française de Belgique-Action de Recherches Concertées” and from the FNRS. M. Landtmeters and S. De Simoni are recipients of a FRIA fellowship.

References

Protective effects of coelenteramine derivatives against uva-mediated cellular damage in skin fibroblasts.

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Coelenteramine (CLM; see figure) is a major oxidation product of imidazolopyrazinone antioxidants such as coelenterazine (de Wergifosse, 2004). CLM affords protection in acellular (de Wergifosse, 2004) as well as in cellular oxidative stress tests (Cavalier, 2001). In this study, we synthesised different CLM analogues to test their protective activities against UVA-induced cellular damages. UVA radiation is well known to interact with cutaneous chromophores like flavins and porphyrines and generate reactive oxygen species (ROS) prone to attack proteins, lipids and DNA of skin cells (Mariethoz,1998). We studied the protection afforded by two CLM analogues bearing a catechol function (JFC54 and JFC58), and two phenol analogues, CD31 and CD22 (see figures), against damages induced by UVA irradiation (25J/cm²) of skin fibroblasts. Production of ROS was followed by the release of dichlorofluoresceine (DCF) in the medium from dichlorofluoresceine diacetate (DCFH-DA) loaded cells, DNA damage was evaluated by COMET assay and the redox status of cells was evaluated by the quantification of glutathione (GSH) content. These three tests were realised just after irradiation. Fibroblasts survival was evaluated by the measure of the release of lactate deshydrogenase in supernatants 24 hours after irradiation. The two catecholic compounds (50µM) revealed to be endowed with efficient protective properties in DCF, COMET and GSH tests, affording a similar protection as trolox applied at 1 mM. CD22 and CD31 were not effective at all. This underlines the necessity of catechol function to confer protection in our stress conditions. Nevertheless, when evaluating the capacity of these two catechol derivatives to improve cell survival, only JFC58 was able to protect cells. JFC54 was not efficient and even seemed to act as a photosensitizer. This effect could be related to the addition of a R2 group as a similar photosensitizing effect was also observed with CD31. In conclusion, JFC58 is a CLM analogue providing good protection against UVA-mediated damage in skin fibroblasts. Experiments are in progress to better understand its mechanisms of action, among which metal chelation could play an important role. Together, these results could have the way towards new compounds active against ageing- and UV-induced skin alterations.

This work was supported by the wallon region. We thank C. De Tollenaere and J.F Cavalier for the synthesis of compounds.

References
Cloning of *Arenicola marina* peroxiredoxin 6

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PRDXs are a family of heme-free and selenium-free peroxidases found in all phyla, from bacteria to plants and animals (WOOD et al., 2003). The catalytic residues of PRDXs are cysteines. To reduce peroxides, the N-terminal conserved peroxidatic cysteine is first oxidized into sulfenic acid which then reacts either with glutathione or with another cysteine in the enzyme.

Here we report the cloning of the homologue of mammalian PRDX6 in the marine worm *Arenicola marina*. This annelid lives in intertidal sediments, facing high hydrogen sulfide (H$_2$S) concentrations (GRIEHSABER AND VÖLKEL, 1998). Moreover, this worm is also exposed to important oxidative threat caused by photochemical reactions as well as by the hypoxia-reoxygenation cycles associated with low and high tides.

Recent work suggests that H$_2$S is not only a toxic gas but that it could also regulate cellular activity thanks to its ambivalent redox properties. Interestingly, it has recently been suggested that bovine PRDX6 could use H$_2$S as electron donor, converting this gas into a less toxic derivative concomitantly to the detoxification of reactive oxygen species (PESHENKO AND SHICHI, 2001). Therefore, we searched for a PRDX6 homologue in *Arenicola marina*, suspecting that such a protein could play important detoxification roles against reactive oxygen species and H$_2$S in this animal. A reverse cloning approach was used based on conserved PRDX6 amino acid sequences among different animal species. Using degenerate primers, a partial cDNA sequence coding for *Arenicola marina* PRDX6 was PCR-amplified. Subsequently, we designed specific primers and obtained the full-length *Arenicola marina* PRDX6 cDNA by RACE-PCR.

The deduced peptidic sequence, besides being highly conserved between several homologous PRDXs in various vertebrate and invertebrate species, revealed the presence of additional cysteines. The existence of these cysteines may be functionally significant and may indicate that these residues could play an important role in the detoxification of H$_2$S in these animals living in sulfide-rich environments, like in the case of the cysteines from the giant hemoglobin found in these worms (ZAL et al., 1997).

Based on the peptidic sequence, tridimensional models of *Arenicola marina* PRDX6 allowed us to postulate several hypothetical enzymatic mechanisms for this protein. Work is in progress to purify and characterize recombinant *Arenicola marina* PRDX6.

We thank F. Zal and X. Bailly from the Marine Biological Station of Roscoff for providing cDNAs of *Arenicola marina*.

**References**


Evolution of serum α-tocopherol in the postprandial and postabsorptive phases in type 1 diabetes mellitus.

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It has been postulated that part of the increased cardiovascular risk in diabetes can be attributed to the oxidative stress arising from abnormalities in postprandial glucose and lipids. In order to investigate this hypothesis in Type 1 diabetes mellitus, twenty-three patients received a standard fat-rich breakfast and lunch. Blood samples were taken at fasting (F), just after the post-breakfast glycemia peak (BP) (identified by continuous subcutaneous glucose monitoring), 3-h postbreakfast (B), just after the post-lunch peak (LP), just after the post-lunch dale (LD) and 5 hours after lunch (L). In contrast to the postprandial increase in serum triglycerides, α-tocopherol decreased from 11.7 ± 3.0 (F) to 10.9 ± 2.3 µg/mL (BP), p = 0.01, and remained low during both postprandial periods (till LD), only to return to levels similar to fasting in the postabsorptive phase (L) 11.2 ± 2.5 µg/mL (p = 0.005). Even when expressed relative to lipids, α-tocopherol decreased from 4.43 ± 0.76 µmol/ mmol lipid at F to 4.12 ± 0.82 at B (p = 0.006) and then returned to 4.43 ± 0.82 µmol/ mmol lipid at L (ns from F). Plasma malondialdehyde increased gradually from 1.02 ± 0.36 (F) to a maximum of 1.14 ± 0.40 µmol/L (LP). In L values reverted to fasting concentrations. These results indicate that the postprandial state in T1DM is characterised by a deficiency in α-tocopherol that is accompanied by increased lipid peroxidation.
Mitochondrial superoxide generation contributes to apoptosis in low-glucose cultured insulin producing beta cells

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High rates of TCA-flux and coupled electron transport activity have been associated with increased mitochondrial superoxide generation, and thus considered as potential cause of cellular dysfunctions and apoptosis (NISHIKAWA et al., 2000). This mechanism has been proposed to be responsible for losses in insulin-producing beta cells under conditions of prolonged hyperglycemia (BINDOKAS et al., 2003). In the present study we have examined whether normal rat beta cells generate ROS under acute exposure to glucose, and whether this is related to the metabolic redox state of the cells. Single beta cells were purified by FACS and then incubated for 60 min at different glucose concentrations before FACS analysis of changes in their NAD(P)H /FADH$_2$ levels (redox state) and their fluorescence for H$_2$DCF-DA-derived (peroxide-like ROS) or dihydroethidium-derived (superoxide) oxidation products. Glucose (from 2.5 to 20 mM) dose-dependently increased NAD(P)H levels with EC$_{50}$ ≈ 8 mM but did not induce peroxide or superoxide formation. On the contrary, an increased ROS and superoxide generation was seen with decreasing glucose concentrations from 5 mM on, and was more marked in beta cell subpopulations with lower metabolic responsiveness to glucose. This low-glucose-induced effect was not observed in islet endocrine non-beta cells which were mainly composed of glucagon-containing alpha cells. Superoxide production at low glucose was suppressed by 1) the electron transport inhibitors rotenone, TTFA and antimycin A, and 2) the mitochondrial fuels leucine (10mM) and succinic acid (methyl ester, 20mM) that also prevented ROS (DCF) production. It was dose-dependently decreased by the SOD mimetic MnTBAP (10-50µM) which was therefore used to assess whether low-glucose–induced superoxide formation is involved in beta cell apoptosis that is known to occur during prolonged exposure to glucose levels under 5mM (HOORENS et al., 1996). We confirmed the apoptotic effect of 3 days culture at 3 mM glucose and observed a dose-dependent protective effect of the SOD mimetic. In conclusion, our data demonstrate that the glucose-induced mitochondrial activity in normal beta cells is not associated with an accumulation of ROS and superoxide radicals. On the other hand, higher superoxide levels were detected at low mitochondrial activity, as present at non-stimulatory glucose concentrations; when sustained, this superoxide production is one of the factors causing apoptosis in beta cells with low metabolic activities.

References

Characterization of mitochondrial markers and transcription factors involved in mitobiogenesis in mtDNA-depleted cells

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Cells totally (rho0) or partially (rho-) depleted in mitochondrial DNA (mtDNA) are commonly used for studying the cellular responses to mitochondrial dysfunction. Despite their chronic energy depletion, these cells still survive and display interesting features. Indeed, mitochondrial structures are still observed, and one can measure a mitochondrial membrane potential in these cells. Therefore, an active mitochondrial biogenesis must be maintained in mtDNA-depleted cells.

In order to further delineate factors involved in the mitobiogenesis in rho0 143B and rho- L929 cells, we first studied the expression level of some nuclear-encoding mitochondrial proteins used as markers. Using CAT reporter system and “real-time” PCR, we show that the β-subunit of the F1-ATPase and COXVb subunit are up-regulated in rho0 143B cells while cytochrome c abundance is unchanged in this cell line. We also show that protein level of TOM40 is elevated in both mtDNA-depleted cell types while importation of mitochondrial proteins in the matrix is reduced. On the other hand, the abundance of mtTFA/TFAM protein, a mitochondrial transcription factor that controls transcription and replication of mtDNA, is reduced in rho 143B cells.

As the activation of numerous transcription factors have been involved in the mitobiogenesis (NRF-1, NRF-2, Sp1, YY1, CREB, PPARγ, MoyD, MEF2α, AP-1…), we thus started to systematically characterize the activation level of these factors and some upstream signaling. To determine abundance, localization and activity of these regulators of gene expression we used DNA-binding assay, luciferase reporter systems, western blotting and immunostaining and found that only CREB (cAMP-responsive element binding protein) and HNF-1 and HNF-4 (hepatocyte nuclear factor-1 and 4) seem to be differentially activated in rho0 cells. Furthermore AMP-activated kinase (AMPK) and Casein Kinase II (CKII) are considered to be “energy sensors” of the cell. We show here, using in vitro kinase assays that AMPK is activated in rho- L929 cells but CKII is not. The AMPK activation has been confirmed by immunostaining of the phosphorylated form on Thr172. The activation of AMPK is not accompanied by an increase of NRF-1 activity, a substrate of AMPK. The implication of these signalling in the mitobiogenesis and metabolic processes is currently under investigation.

T. Arnould is a Research Associate of FNRS (Fonds National de la Recherche, Brussels, Belgium) and L. Mercy is a recipient of a FRIA (Fonds pour la Recherche Scientifique dans l’Industrie et l’Agriculture) fellowship.
Characterization of ThOXs, the thyroid NADPH oxidases, as components of the thyroid $H_2O_2$ generating system and differentiation marker.

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In the thyroid the iodination of thyroglobulin and oxidative coupling of the resulting iodotyrosines is catalyzed by the thyroperoxidase. Both reactions are dependant on the presence of $H_2O_2$ as an electron acceptor. Molecular identity of the corresponding $H_2O_2$ generating system in the thyroid has been elucidated by the cloning of two thyroid cDNAs encoding two new members of the NADPH oxidase family (ThOX1 and ThOX2) based on their functional homology with the leukocyte $H_2O_2$ generating system. The role of ThOXs in iodide organification is strongly supported by the finding of homo- and heterozygous nonsense mutations in ThOX2 of patients with total and partial organification defect respectively. ThOXs are co-localized with TPO at the apical pole of the thyrocytes. Messenger RNA as well as protein expressions are stimulated by the cAMP pathway in the dog thyroid but not in human thyroid. Human ThOX1 and ThOX2 genes are arranged in a head to head configuration on chromosome 15q15.3 and are separated by a 16kb-long region. The promoters of both genes do not resemble each other and differ from promoters of other known thyroid specific genes. No cAMP responsive element has been found in any of human ThOX promoter and functional studies have shown that these promoters are not positively controlled by cAMP.

ThOX protein expression as differentiation marker has been studied during mouse thyroid development. Using the immunohistochemical approach with the antibody recognizing both ThOX1 and ThOX2 we found ThOX proteins expressed from the embryonic day 15.5, at the same embryonic stage as the sodium-iodide symporter (NIS) and one day after thyroglobulin. Adult mice lacking functional TSH receptor (hyt/hyt) were depleted of ThOX specific immunostaining suggesting that the functional TSH/TSHR axis is necessary for the expression of ThOX proteins in the same manner that it is required for the expression of NIS and thyroperoxidase. Using the same methodology we also studied the expression of ThOX proteins in a mouse model of papillary thyroid carcinoma (RET/PTC3). Although NIS expression is strongly decreased already at the age of 1 month the expression of ThOX proteins is preserved in the apical membrane of the follicular regions of the thyroid but disappears at later stage of tumor progression (18 months).

This work was financially supported by a grant of the”FRSM” and the ”Fondation Van Buuren”.

Modulation of DNA repair: preliminary comet assay study of some flavonoids after alkylating DNA damage

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The flavonoids constitute a highly investigated group of secondary metabolites particularly abundant in our diet. Many of these compounds present a number of biological properties, including antioxidant and carcinostatic activities; some are known to modulate the activity of various enzymes¹ and could be expected to interfere in DNA repair mechanisms. Previous studies using H₂O₂ as the DNA damaging agent tend to show that some flavonoids effectively modulate DNA repair², but an antioxidant effect cannot be entirely ruled out.

The present work was then undertaken using an alkylating agent to damage DNA and probe repair.
P388D1 cells were exposed to ethyl methyl sulphonate (EMS) (2 mM) for 3 h, washed with PBS (time 0) and maintained up to 48 h either in culture medium (control) or in culture medium supplemented with flavonoids from 5 different chemical classes, quercetin (8 µM), chalcone (8 µM), apigenin (2 µM), epicatechin (16 µM), diosmin (24 µM) or sakuranetin (16 µM). The kinetic of repair was investigated after 12, 24 and 48 hours by the alkaline comet assay, a measurement of DNA single strand break (ssb) level (Olive tail moment and tail DNA).

After 12 h exposure to quercetin and chalcone, DNA breakage was found to be increased compared with control; after 24 h, it was found to be decreased, indicating faster excision and repair. For apigenin, epicatechin, and diosmin, the ssb were constantly lower than those of control cells, pointing to a faster overall repair. Sakuranetin showed an inverse effect, with higher levels of ssb at 24 h.

These preliminary data have to be confirmed, notably by investigating the mechanisms involved, but suggest that some major dietary compounds may promote DNA repair, which could help to prevent the carcinogen risk.

References
Peroxiredoxin-Glutaredoxin Fusion Proteins – An Amalgamation of Reducing Power

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For those working in the field of oxidative stress research, peroxiredoxins are no complete strangers. These all-pervading thiol-dependent redox proteins constitute a large family of antioxidant enzymes whose peroxidase activity is manifested by the presence of a nucleophilic cysteine residue. It is this residue which, following reduction of the hydroperoxide, condensates with another thiol group into a disulfide bond. Subsequent reduction of this form was initially thought to occur exclusively by thioredoxin. However, recent characterization of glutaredoxin-reducible peroxiredoxins in organisms such as *Populus* and *Schistosoma* have broadened the notion that, besides thioredoxin, additional reducing partners are possible. Lately, much attention has been paid to the characterization of a novel class of fusion proteins that combine a peroxiredoxin and a glutaredoxin entity into one protein. At our laboratory, this resulted in the identification two representatives in *Haemophilus influenzae* and *Chromatium gracile*. Apart from these, this type of hybrid enzyme has yet been documented only in the bacteria *Neisseria* and *Vibrio*.

Peroxiredoxin-Glutaredoxin (or PGdx) enzymes catalyze the glutathione-dependent reduction of hydroperoxides. This is made possible by the mutual interactions occurring between both domains. Their catalytic efficiencies approximate the ones of their separate counterparts, sometimes accompanied by abnormal kinetic behavior. Besides a biochemical characterization of *H. influenzae* PGdx we are also studying the physiological implications of the enzyme, as well as the regulatory aspects concerning its expression. From these studies we hope to gain a better understanding of these hybrid peroxidases and to get insight into the behavior of *H. influenzae* when exposed to oxidative stress.

F. Pauwels is recipient of a grant by the “Institute for the Promotion of Innovation through Science and Technology in Flanders” (IWT Vlaanderen, grant 3072).

References

Proteomic, transcriptomic, signal transduction and functional studies of stress-induced premature senescence (SIPS) & potential role of SIPS in human ageing

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SIPS occurs in several proliferative cell types after subcytotoxic exposure to tert-butylhydroperoxide (t-BHP), H$_2$O$_2$, ethanol, UV-B, etc. TGF-β1 overexpression triggers the induction of several biomarkers of replicative senescence (RS) in human diploid fibroblasts (HDFs) within 72 hrs after H$_2$O$_2$ or UV-B stress. H$_2$O$_2$-induced p38$^{\text{MAPK}}$ phosphorylation triggers a sustained overexpression of TGF-β1 through a sustained activation of ATF-2 transcription factor, due to establishment of a regulatory loop between TGF-β1 overexpression and sustained p38$^{\text{MAPK}}$ phosphorylation. At 24 hrs after stress, ATF-2 interacts with hypophosphorylated retinoblastoma protein, which allows the biomarkers of RS to appear (FRIPIAT et al., 2002, CHEN et al., 2000, FRIPIAT et al., 2001, CHAINIAUX et al., 2002). H$_2$O$_2$- and UVB-induced SIPS also takes place in HDFs with telomerase activity. Very limited mean telomere shortening is observed in these conditions (MAGALHAES et al., 2002). High resolution 2-D gels and mass spectrometry allowed to identify 30 proteins differentially expressed in SIPS induced by ethanol and t-BHP and/or in RS (DIERICK et al., 2002). Lastly we performed transcriptomic studies with dedicated low density cDNA arrays, and we started functional studies for investigating the role of the genes that are differentially expressed in SIPS and/or RS. These studies allow to hypothesise a potential role of SIPS in in vivo aging.

O. Toussaint is research associate of the FNRS, Belgium. We thank the European projects “Functionage” (contract # QLK6-2001-00310) and “CRAFT-Cellage” (contract # CRAFT-1999-71628), the Région Wallonne projects “Modelage” and “TOXISIPS” and the Région Wallonne/FSE project “Arrayage” (EPH 3310300 R0472/215316)

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The oxidative burst induced by heavy metals in *Nicotiana tabacum* BY2 cell cultures.

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One of the earliest responses of a plant cell under stress conditions is the oxidative burst: a rapid production of huge amounts active oxygen species (AOS) such as superoxide and hydrogen peroxide. The mechanism of this stress-induced AOS-production has not yet been completely resolved. Two major hypotheses present the possible origins of AOS in the oxidative burst: the action of an NADPH-oxidase system - analogue to that of animal phagocytes and/or a pH-dependent generation of hydrogen peroxide by cell wall peroxidases (Wojtaszek, 1997).

In this study we compare the production of AOS in tobacco BY2 cell cultures after treatment with different heavy metals such as Cu, Zn, Al, Pb and Cd. The detection of the AOS was based on the oxidation of aminoantipirin (1 mM) in the presence of 3,5-dichloro-2-hydroxybenzene sulfonic acid (10 mM) (Van Gestelen et al., 1998).

The results show that incubation of the cells with Zn, Al and Pb could not induce a rapid increase in the synthesis of active oxygen species. Nevertheless, stress-induction of tobacco BY2-cells through copper as well as through cadmium results in an oxidative burst. A rapid and concentration-dependent generation of oxygen radicals was apparent within a few minutes after the treatment with concentrations as low as 10 µM Cu or 250 µM Cd. This specific and early response towards copper or cadmium stress was shown to be extracellular. The burst induced by both metals has a very similar kinetic pattern, although differences could be observed in the AOS-production system. Pretreatment of the cell cultures with the NAD(P)H-oxidase inhibitors diphenylene-iodide (2-10 µM) and quinacrine (1-5 mM) prevented the generation of hydrogen peroxide under copper stress for 90%. The inhibitory effect on the Cd-induced burst was less effective (60%). This suggests a different role for the NADPH-oxidase in the AOS-production under Cu or Cd stress.

The strong influence of the pH of the medium on the rate of AOS-production could be an indication for a possible role for the pH-dependent cell wall peroxidases in the Cu and Cd induced oxidative burst. The importance of Ca²⁺ in the signaltransduction pathway to induce the AOS-producing system was tested by blocking the calcium channels in the plasmamembrane with LaCl₃ (0.50 mM) and the use of the calcium chelator EGTA (0.25 mM). Both treatments resulted in an almost complete inhibition of the AOS-production.

It is our intention to further investigate the reaction mechanisms underlying the heavy metal induced oxidative burst in tobacco BY2-cell cultures.

References
Regulation of IL-1β and IL-6 mRNA stability by their 3'-untranslated region.

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Interleukin-1β and -6 are barely expressed in human dermal fibroblasts in vitro. Blocking protein synthesis by cycloheximide results in a large increase of the expression of their mRNA, suggesting that the silencing of these genes is under an active control requiring protein neosynthesis. Selective destabilization of short-lived mRNAs as those of proto-oncogenes and cytokines is facilitated by an AU-rich element (AURE) located in their 3'-untranslated region (3'UTR). This AURE contains one or more AUUUA motifs, which may be overlapping or dispersed. Our work aimed at studying the regulation operated by the AURE containing 3'UTR of IL-1β and IL-6. WI-26, a human embryonic lung fibroblastic cell line, were transfected using constructions containing the Secreted Alkaline Phosphatase (SEAP) as reporter gene under the control of the SV40 promoter (pSV40) and followed by the IL-6 (3'IL6), the IL-1β (3'IL1) or the SV40 (3'SV40) 3'-untranslated region. A maximal secretion of SEAP was measured in the WI-26/pSV40-SEAP-3'SV40 reflecting the strong activity of the SV40 promoter and the lack of AURE in the 3'SV40. By replacing the 3'SV40 by the 3'IL6 or the 3'IL1, an almost complete suppression of SEAP production was observed at both protein and mRNA levels, pointing to the efficacy of the mRNA destabilization activity of the 3'UTR of both cytokines. Similar data were obtained in HEK293, a human embryonic kidney cell line. To determine the importance of the various AUUUA motifs of the 3'UTR of IL-1β and IL-6 in the destabilization of their mRNA, plasmids containing the SEAP gene under the control of an inducible promoter (Ind) and followed by several native or truncated 3'UTR were constructed. They were transiently transfected in HEK293 cells stably expressing a receptor (VgRXR) enabling activation of the Ind promoter by ecdysone or analogs as ponasterone A. Transcription of SEAP was induced by ponasterone A for 24 hours and switched off after washing out of the inducer, and the SEAP activity was measured after another 24 hours. We observed that SEAP activity was largely expressed in pInd-SEAP-3'SV40 transfected cells while it disappeared when all the AUUUA motifs of the IL-1β or IL-6 3'UTR were present. Progressive deletion of the AUUUA motifs resulted in progressive recovery of SEAP expression, showing that the various motifs cooperate to control the mRNA destabilization.
Construction of a set of vectors allowing inducible production of siRNA in Schizosaccharomyces pombe.

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RNA interference (RNAi) is a sequence-specific gene silencing mechanism. It is induced by the formation of dsRNA that are recognised by the Dicer complex and processed into 21-23 long oligonucleotides called siRNA (short interfering RNA). Subsequently, RISC (RNA-Inducing Silencing Complex) binds siRNA that targets the complex towards its homologous mRNA (DYKXHOORN et al., 2003) which is eventually degraded. In contrast to budding yeast, the entire pathway is conserved in the fission yeast Schizosaccharomyces pombe, making it a valuable organism to both study physiological RNAi and to use it as a inducible gene knock-down tool.

In an attempt to apply this method in the fission yeast, we are using three different approaches to produce siRNA. In each case, a vector containing a regulatable promoter activated in presence of tetracycline (tTA’) (GOSSEN et al., 1995) is generated and the ura4 marker required for growth on medium lacking uracil serves as reporter. First, a vector expressing the full length antisens RNA of ura4 (800 nucleotides) (RAPONI AND ARNDT, 2003) is used. Second, we are trying to generate much shorter dsRNA where both strands are linked by either a short hairpin of 25 nucleotides (BRUMMELKAMP et al., 2002) or a longer one of 350 nucleotides (SCHRAMKE AND ALLSHIRE, 2003). The ability of these different dsRNA to induce silencing of ura4 will be presented.

A. G. and L. T. are recipient of a FRIA fellowship, DH is supported by the Human Frontier Science Program (Long term fellowship LT00665/2003). This work is supported by a FNRS grant (Convention FRFC 2.4504.00-2003).

References
**LDL oxidation by the Myeloperoxidase / H₂O₂ / Cl⁻ system is inhibited by various thiol-containing molecules**

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The Myeloperoxidase (MPO) / H₂O₂ / Cl⁻ system is involved in the production of HOCl by neutrophils and monocytes during the inflammatory process. Furthermore, it contributes to LDL oxidation, a pathogenic factor in the development of atheromatic lesions. Some of us recently developed an ELISA system to specifically measure the product of this reaction. The results illustrate a reproducible oxidation of the apoprotein-B100 due to the coupling between MPO and LDL before the enzyme is oxidized with H₂O₂.

The present study documents the effects of thiol-containing molecules such as glutathione (GLU), captopril (CAP) and N-acetylcysteine (NAC) and its lysinate salt (NAL) on LDL oxidation by the MPO system. The extent of inhibition was examined in presence and in absence of LDL. The quantity of methionine required to inhibit 50 % of the thiol oxidation by the MPO system was first determined and the inhibition of LDL oxidation in relation to the concentrations of the different investigated molecules (5 to 300 μM) was then measured by the ELISA test.

The results indicate that GLU (IC 50 % = 287 ± 9 μM, n = 5) inhibits more intensively the MPO system than NAL (107 ± 6 μM, n = 5) and NAC (101 ± 9 μM, n = 5), which themselves are more efficient than CAP (58 ± 1 μM, n = 5). This is consistent with some literature data on the ability of these last molecules to interact with HOCl and H₂O₂. As far as LDL oxidation is concerned, 5 μM of thiol-containing molecules show an inhibition of 32 ± 9 % for GLU, 47 ± 5 % for CAP, 53 ± 13 % for NAL and 61 ± 13 % for NAC. In addition, 30 μM of these compounds give an inhibition of 82.8 ± 1 % for GLU, 85 ± 3 for CAP, 90 ± 3 % for NAL and 87 ± 4 for NAC.

In conclusion, thiol-containing molecules, which have clearly documented antioxidant properties against oxygen-derived species including HOCl, are also good scavengers of the MPO system (GLU > NAC ~ NAL > CAP). Furthermore, they inhibit the LDL oxidation despite the coupling between MPO and LDL (NAL ~ NAL > CAP > GLU). However, this inhibition cannot be explained only by their interaction with HOCl and further studies on the reaction mechanism are necessary.
Transferrin glycation and iron isoforms

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In diabetes, protein function is altered by glycation, but the impact on the Fe³⁺-binding function of transferrin (Tf) is largely unknown. Since free Fe²⁺ is capable of stimulating free radical reactions, the sequestration by transferrin is a very important antioxidant mechanism in human plasma.

We aim therefore to investigate the effects of glycation on the distribution of Fe³⁺ on the two Fe³⁺-binding sites of Tf. First, Tf was glycated in vitro by pre-incubation with different concentrations of glucose for 14 days. Loading with Fe³⁺-compounds resulted in theoretical Tf Fe³⁺-saturations of 32%, 64% and 96% (monitored by spectrophotometry). Fe³⁺ Tf-isoforms were separated by iso-electric focusing. Fe³⁺-binding was highest when incubating with Fe:nitritotriacetate and reached a steady state overnight. Increasing the Fe³⁺-load resulted in a shift of isoform profile towards the diferric form: in freshly prepared Tf, Fe2-Tf represented 6.3%, 30% and 66% of all isoforms at 32%, 64% and 96% theoretical Fe³⁺-saturation respectively. Fe³⁺ was equally distributed to the Fe1N- and Fe1C-Tf isoforms. Glycation decreased binding of Fe³⁺ to Tf (monitored at 450nm). At low theoretical Fe³⁺-saturation (32%), glycation increased the proportion of Fe2-Tf (18 ± 3% after 33.3 mmol/L glucose versus 12 ± 4% after 0 glucose, p=0.01). In contrast, at 96% theoretical Fe³⁺-saturation, Fe2-Tf decreased linearly with increasing glycation (r=0.97, p=0.008).

Pre-incubation, independently of glycation, favoured the Fe1N-Tf isoform at 64% theoretical Fe³⁺-saturation (27 ± 0.7 versus 23 ± 1.1% of the Fe1C-Tf isoform, p=0.009).

We conclude that glycation may impair Fe³⁺-binding and affect Fe³⁺-Tf-isoform distribution depending on the concentration. The diagnostic implications of these results on the oxidative status of diabetici need further elucidation in clinical studies.
Prokaryotic glutathione metabolism revisited: its role in peroxide stress resistance.

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In previous reports, we have shown that the tripeptide glutathione (γ-Glu-Cys-Gly) is an important antioxidant during aerobic growth of *Haemophilus influenzae* (VERGAUWEN et al., 2003a, VERGAUWEN et al., 2003b). It thus was a surprise to establish that *H. influenzae* acquires glutathione by import instead of *de novo* synthesis, as is the case for other γ-proteobacteria such as *Escherichia coli*. As such, the glutathione metabolism of *H. influenzae* differs from that of other γ-proteobacteria, for which *de novo* synthesized glutathione is accepted to be dispensable for aerobic growth (GREENBERG & DEMPLE, 1986).

To accentuate the importance of glutathione for *H. influenzae* cells grown under aerobic conditions, we here show that the intracellular glutathione content increased 1.5-fold after anaerobically growing cells were placed in air. The intracellular glutathione content of a catalase mutant was consistently higher compared to the isogenic parent under anaerobic as well as under aerobic growth conditions. In contrast, glutathione biosynthesis is not regulated by oxidative stress in *E. coli*.

To explain the apparent differences in relation to the function of glutathione among γ-proteobacteria, we provide evidence that the antioxidant function of glutathione in *H. influenzae* entirely depends on the presence of the thiol peroxidase called PGdx. PGdx is a fusion protein composed of an N-terminal peroxiredoxin-like domain, followed by an amino acid stretch that is homologous to glutaredoxins.

By constructing and phenotypically characterizing *H. influenzae* strains deficient in either PGdx, catalase or both antioxidant enzymes, we were able to show that i) no other enzyme than the PGdx peroxidase scavenges peroxides in a glutathione-dependent manner, ii) PGdx is the principal scavenger of respiratory-generated *H₂O₂* during log-phase growth, iii) catalase becomes of predominant importance when the cytoplasm is subjected to a *H₂O₂*-flux which exacerbates that generated by the own respiratory metabolism, and iv) PGdx can be regarded as the functional counterpart of the well-known bipartite peroxidatic system AhpCF of enterobacteria.

References
Enhancement of quinone redox cycling by ascorbate leads to cancer cell death through a caspase-3 independent pathway. An in vitro comparative study.

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Since the higher redox potential of quinone molecules has been correlated with enhanced cellular deleterious effects, we studied the ability of the association of ascorbate with several quinone derivatives (having different redox potentials) to cause cell death. The rationale is that the reduction of quinone by ascorbate should be dependent on the quinone redox potential thus determining if reactive oxygen species are formed in more or less amount, leading ultimately to cell death or cell survival. We observed a close relationship between standard redox potentials, oxygen uptake, free ascorbyl radical formation and cell survival. The oxidative stress induced by the mixture of ascorbate and the different quinones decreases cellular contents of ATP and GSH while caspase-3-like activity remains unchanged. Again, a close relation was observed between extreme values of half-redox potential of quinones and their effects on ATP and GSH when they were associated with ascorbate. It seems that the strong ATP depletion induced by these associations, may reflect an impairment in the glycolytic pathway. Such a drop in ATP content may also explain the lack of activation of caspase-3. In conclusion, our results indicate that the cytotoxicity of the association quinone/ascorbate on cancer cells may be predicted on the basis of half-redox potentials of quinones.

J. Verrax is recipient of a FRIA fellowship.
Effect of age on chlorzoxazone biotransformation in male Wistar rat liver microsomes: role of oxidative stress.

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Biotransformation of a drug and, therefore its therapeutic effect, may be modified during ageing. Among different causative factors, the impairment of normal cellular functions by free radicals has been evoked as playing a critical role. The aim of our study was to evaluate the effect of age on the expression and activity of CYP2E1. In addition, we determined some markers of oxidative stress like TBARS (thiobarbituric acid reactive substances), histochemical detection of aldehydes, carbonyl proteins, GSH content and antioxidant enzyme activities (superoxyde dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH px)). Liver microsomes were prepared from male Wistar rats of 3, 9 and 24 months old. Chlorzoxazone was used as a probe to assess CYP2E1 activity. Metabolite formation, 6-hydroxy-chlorzoxazone, was quantified by HPLC. CYP2E1 protein content was determined by Western Blot and mRNA level by RT-PCR. Lipid peroxidation was assessed following the formation of TBARS and by histochemical detection of aldehydes, the direct Schiff’s reaction. The protein carbonyl content was determined by measuring at 355 nm the formation of hydrazone after reaction with DNPH (diphenylhydrazine) and by immunoblotting after derivation with DNPH (dinitrophenylhydrazine). GSH levels were determined fluorimetrically by measuring the thiol content by reaction with OPT (o-phtaldialdehyde). SOD activity was measured by the standard method using generation of superoxyde anion by xantine oxidase and measurement NBT (nitroblue tetrazolium) reduction. Catalase activity was measured with the TiSO4 method and GSH peroxidase by recording the NADPH oxidation. Results revealed that chlorzoxazone hydroxylation (CYP2E1 activity) increased from 3 to 9 months, and then decreased significantly between 9 and 24 months. Interestingly, CYP2E1 microsomal protein followed the same profile from 3 to 9 months, but remained constant thereafter. Levels of CYP2E1 mRNA did not change over the whole period. While the amount of proteins did not change, their functionality may be affected by oxidative stress (significant increase in thiobarbituric acid reactive substances and decrease in reduced glutathione level). However, no changes in carbonyl protein content and antioxidant enzyme activities were observed. The decrease in CYP2E1 activity in rats at 24 months is most probably due to post-translational modifications of CYP2E1 proteins. Indeed, it may be correlated with an accumulation of oxidative damage.
Thiamine triphosphate, a new signal involved in response to stringent conditions in bacteria and eukaryotes?

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In most organisms, the main form of thiamine is the coenzyme thiamine diphosphate. Thiamine triphosphate (ThTP) is present in low amounts in most organisms from bacteria to mammals (1). ThTP may phosphorylate certain proteins (2), but its biological role remains unknown. In Escherichia coli, ThTP appeared only under particular circumstances. When grown aerobically in LB medium, E. coli contained no detectable amounts of ThTP, but when they were transferred to M9 minimal medium supplemented with a suitable carbon source, there was a rapid but transient accumulation of relatively high amounts of ThTP (about 20% of total thiamine). Carbon sources leading to a rapid synthesis of acetyl-CoA (glucose, pyruvate, gluconate…) were most effective in inducing a rapid accumulation of ThTP. Other compounds, such as Krebs cycle intermediates, were ineffective. When a mixture of amino acids was present in addition to the carbon source, ThTP accumulation was impaired, suggesting that the latter may occur in response to amino acid starvation. In order to test the importance of ThTP for bacterial growth, we used an E. coli strain overexpressing a specific human recombinant thiamine triphosphatase (3) as a GST fusion protein. Those bacteria were unable to accumulate measurable amounts of ThTP. In minimal medium supplemented with glucose or pyruvate, after a few hours of normal initial growth, they exhibited an intermediate plateau in growth rate compared to control bacteria expressing GST alone or a GST fusion protein unrelated to thiamine metabolism. This is the first demonstration of a physiological role of this ubiquitous compound in any organism (4). These results suggest that ThTP synthesis is part of a reaction cascade involved in the adaptation of bacteria to stringent conditions such as amino acid starvation. ThTP may play a role in stress response in other organisms too. Indeed, preliminary observations have shown that leaves from Arabidopsis thaliana produce ThTP during withering (1), while cultured neuroblastoma cells accumulate ThTP upon acidification.

B. Wirtzfeld is recipient of a FRIA fellowship. P. Wins is Research Associate and L. Bettendorff is Senior Research Associate at the FNRS.

References
Oxidation of low-density lipoproteins by myeloperoxidase at the surface of endothelial cells: an additional mechanism to subendothelium oxidation.


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Atherosclerosis is generally viewed as a chronic inflammatory disease of the arterial intima characterized by the formation of the atherosclerotic plaque. According to the oxidation hypothesis, low-density lipoproteins (LDL) are trapped and retained in the intima where they undergo oxidative modification (Berliner et al., 1997). Myeloperoxidase (MPO), an abundant enzyme secreted from activated neutrophils, and monocytes, may be involved in the development of coronary artery disease (Zhang et al., 2001). Indeed, the MPO promotes oxidative damage of host tissues at sites of inflammation, including atherosclerosis lesions. While MPO has been implicated as an enzymatic catalyst of LDL oxidation, in the intima, converting the LDL into a high-uptake form for macrophages, we hypothesize and try to show that this oxidation of LDL might also occur at the surface of the endothelial cells under physiological conditions.

Ea.hy926 endothelial cells were incubated for 24h in a DMEM medium containing native LDL at concentrations ranging from 500 to 2000µg/ml, in the presence or absence of 2.5 µg/ml of recombinant-MPO. The production of MPO-oxidized LDL (Mox-LDL) was measured by an ELISA assay. The incubation of endothelial cells with native LDL in conjunction with MPO resulted in a 1.5 to 2.5-fold stepwise increase in Mox-LDL production (ANOVA, p<0.001), while at a constant concentration of native LDL (1500 µg/ml), MPO significantly increased the production of Mox-LDL (2 to 2.6-fold increase in comparison with the controls, p<0.001) in a dose-response effect of MPO concentration ranging from 0 to 3µg/ml. Meanwhile, the co-incubation of native LDL (1500 µg/ml), with MPO (2 µg/ml) in the absence of endothelial cells showed Mox-LDL production that is significantly lower than that in presence of cells.

In this study we tried to show that a cellular mechanism of LDL oxidation by myeloperoxidase already occurs at the surface of the endothelial cells and may not be restricted to the sub-endothelial space. The serum levels of both LDL and myeloperoxidase, as well as the presence of endothelial cells, seem to play interactive roles in Mox-LDL genesis. Our findings seem to support this hypothesis and are consistent with the previously described relationship between blood, MPO concentrations and coronary artery disease (Zhang et al., 2001).

References
