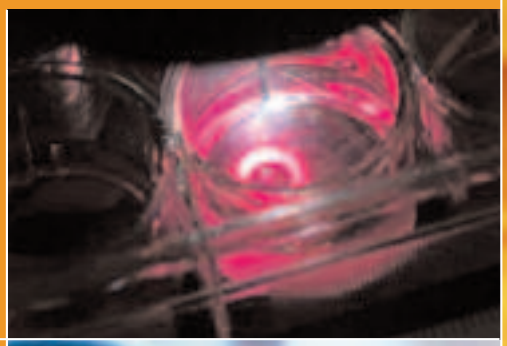
THEMATIC PROJECTS

FAPESP
THE STATE OF SÃO PAULO
RESEARCH FOUNDATION

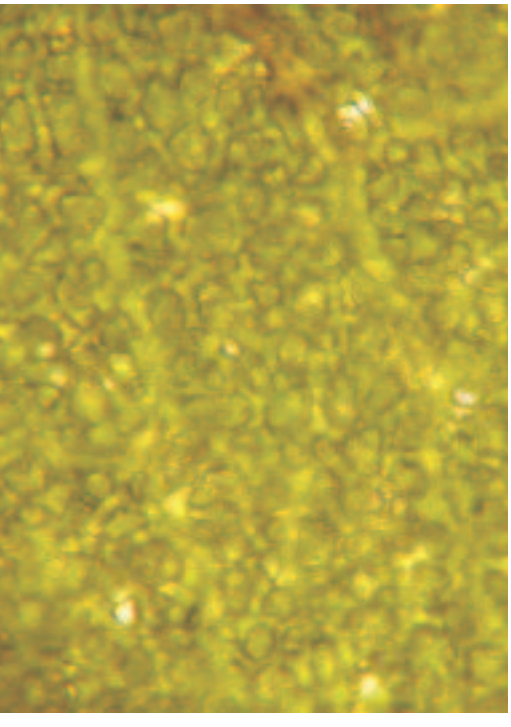
OPPORTUNITIES FOR HEALTH RESEARCH IN BRAZIL

BIOCHEMISTRY

BIOPHYSICS



EXPLORATORY RESEARCH RECOGNIZED WORLDWIDE



Cancer, genetics, immunology, biochemistry, tropical diseases, medicine. In these and many other sub-areas of Health science, Brazilian scientistas contributed results recognized worldwide.

FAPESP, the State of São Paulo Research Foundation, is one of the main Brazilian agencies for the promotion of research. The foundation supports the training of human resources and the consolidation and expansion of research in the state of São Paulo.

Thematic Projects are research projects that aim at world class results, usually gathering multidisciplinary teams around a major theme. Because of their exploratory nature, the projects can have a duration of up to four years.

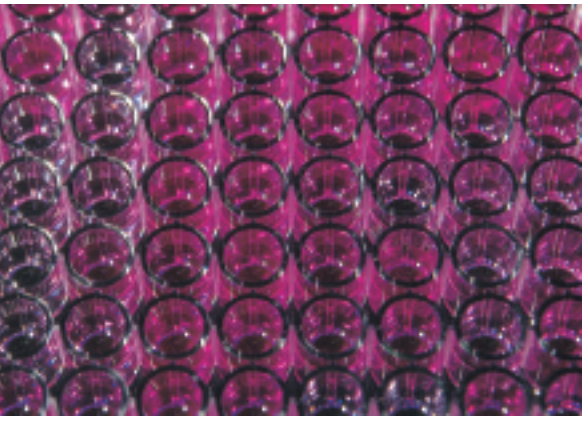
SCIENTIFIC OPPORTUNITIES IN SÃO PAULO, BRAZIL

Brazil is one of the four main emerging nations. More than ten thousand doctorate level scientists are formed yearly and the country ranks 15th in the number of scientific papers published.

The State of São Paulo, with 40 million people and 34% of Brazil's GNP responds for 53% of the science created in Brazil. The state hosts the University of São Paulo (USP) and the State University of Campinas (Unicamp) , both classified among the 200 best in the world by the Times Higher Education Suplement (THES), the growing University of The State of São Paulo (UNESP), Federal University of ABC (ABC is a metropolitan region in São Paulo), Federal University of São Carlos, the Aeronautics Technology Institute (ITA) and the National Space Research Institute (INPE).

Universities in the state of São Paulo have strong graduate programs: the University of São Paulo forms two thousand doctorates every year, the State University of Campinas forms eight hundred and the University of the State of São Paulo six hundred.

In addition to the three state universities the state has 19 research institutes, three federal universities of international research level and most of Brazilian industrial R&D. The state houses more than 10 thousand fulltime faculty and 130 thousand students. São Paulo alone, produces more scientific papers than any country in Latin America, except for Brazil.



FAPESP: SUPPORT FOR RESEARCH IN SÃO PAULO

The State of São Paulo Research Foundation (FAPESP) promotes scientific research in the State of São Paulo, Brazil. Through a robust program of fellowships and research grants it supports fundamental and applied research.

Created in 1962, the foundation is entitled by the State Constitution to 1 per cent of the tax revenues of the state of São Paulo. FAPESP has a sizable endowment and has already supported, over these 46 years, 89,000 fellowships and 80,000 research awards.

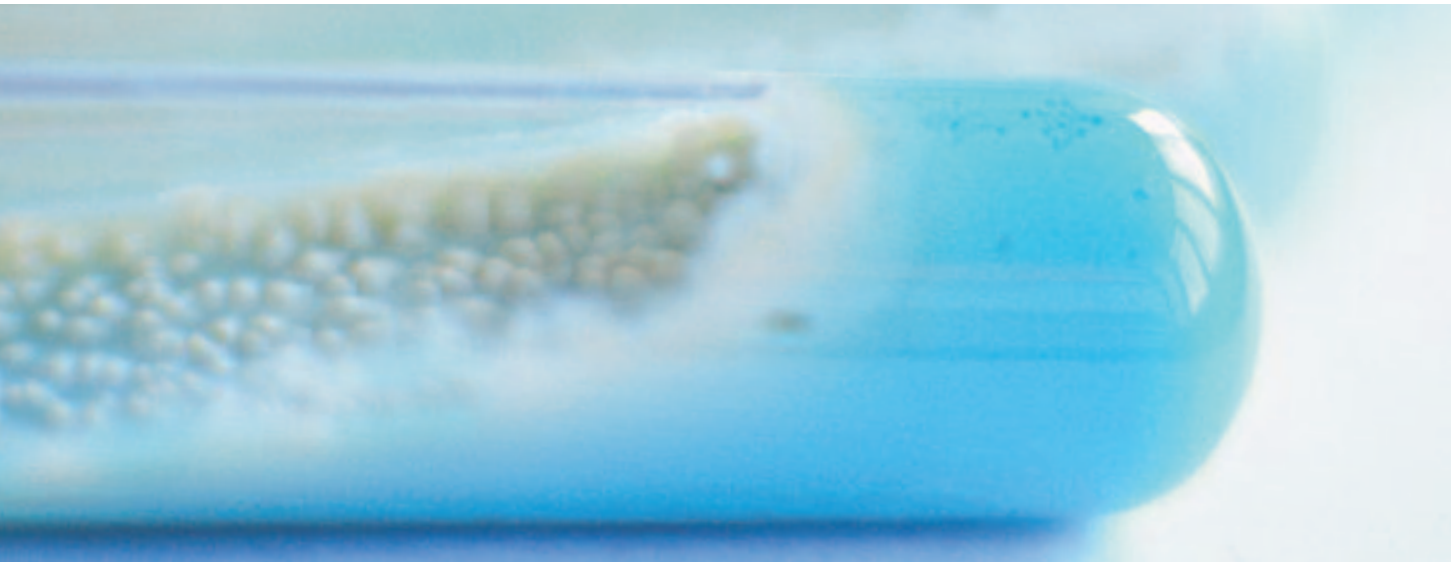
In 2008 FAPESP will invest US$ 388 million in fellowships and research grants. The success rate for proposals in the fellowship programs ranges from 40 per cent to 63 per cent. In the grants programs the proposal success rate ranges from 40 per cent to 60 per cent, depending on the particular type of grant.

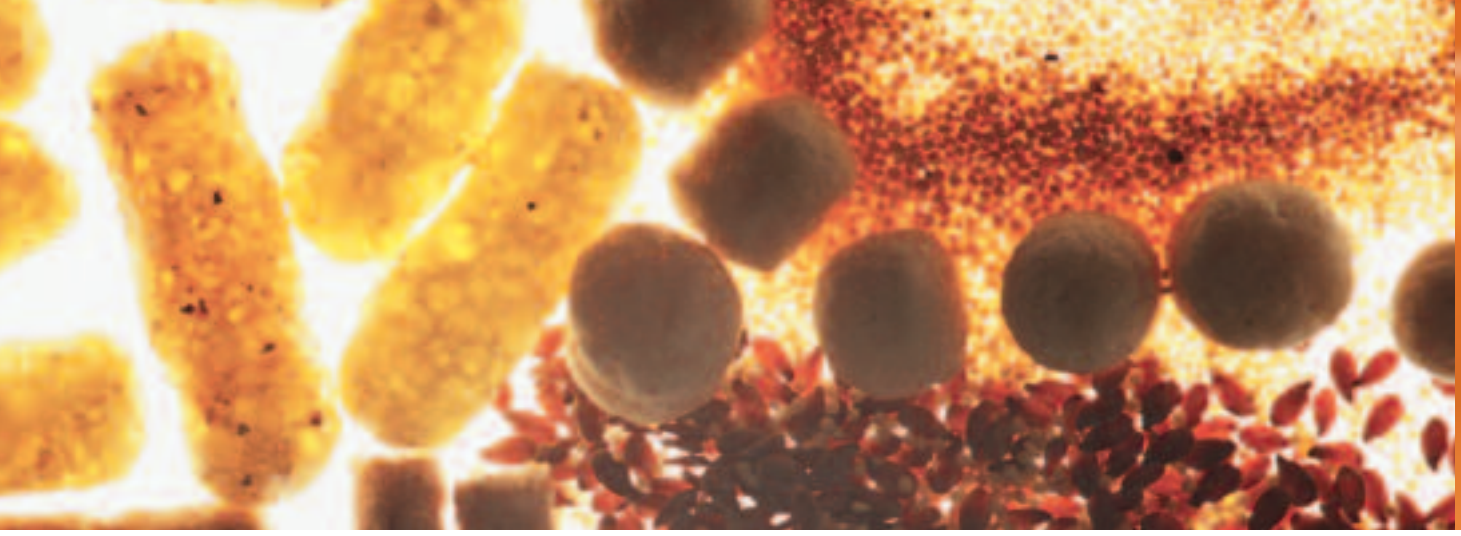
OPPORTUNITIES AND CHALLENGES

One of FAPESP's goals is the broadening and diversification of the research system in the state of São Paulo, strengthening the existing centers of excellence, by supporting their research, and stimulating the creation of new centers or research groups tackling new lines of activity. This is achieved mainly by funding Young Researchers Awards, the Biota-FAPESP Program, RIDC (Research, Innovation and Dissemination Centers) Program and the Thematic Projects.

All of these have in their teams, in addition to experienced scientists, young researchers as post-doctoral fellows, from Brazil and from abroad. FAPESP supports more than one thousand post-doctoral fellowships.

Contact FAPESP (www.oportunidades.fapesp.br) or a coordinator from the Thematic Project which interests you and see how to obtain a post-doctoral internship.





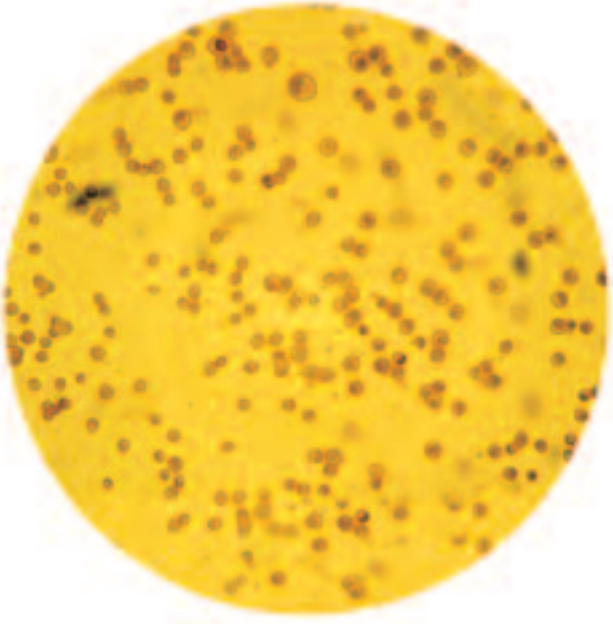
RESULTS OF GREAT IMPACT

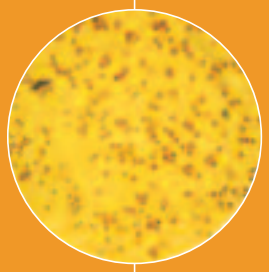
When the research program for Thematic Projects was created, in 1990, FAPESP's objective was to provide a qualitative leap in Brazilian scientific research and meet the state of São Paulo's own particular demands for development. Since then, 1,100 projects in all fields of knowledge have been selected and supported. Selection is through a stringent peer reviewing process, using multiple reviewrs for each proposal.

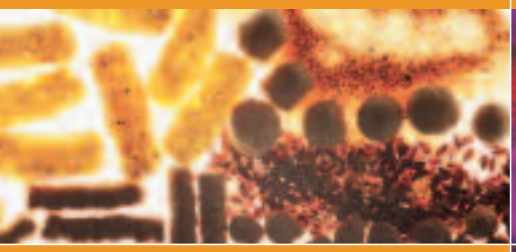
Thematic Projects are characterized by the breadth of their research and the boldness of their objectives. They are supported for four years (as opposed to two years for a regular research grant) and are lead by teams of experienced researchers.

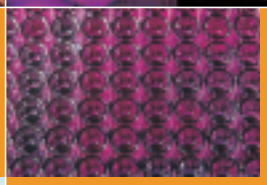
Thematic Projects are funded, on the average, with 450 thousand dollars, plus fellowships. The salaries for the investigators and staff are not included in this amount since in Brazil they are paid by their universities. Each project is lead by 3 PI's and involves several undergraduate and graduate students.

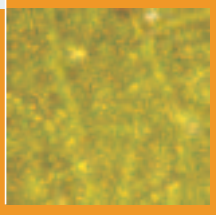
Thematic Projects create opportunities for scientists in São Paulo to advance knowledge by creating internationally competitive science, while, simultaneously, educating a new generation of researchers.











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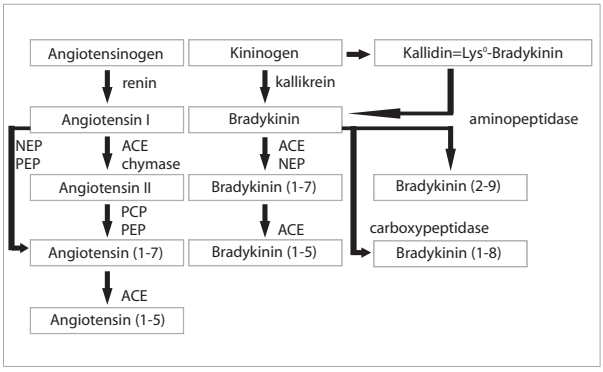
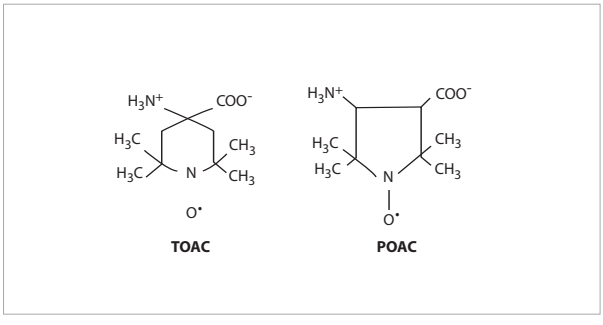
SYNTHESIS AND STUDIES OF PEPTIDES AND ANALOGUES MAINLY INVOLVED IN THE RENIN-ANGIOTENSIN AND KALLIKREIN-KININ SYSTEMS

Clovis Ryuichi NAKAIE

Federal University of São Paulo (Unifesp)



The renin-angiotensin and kallikrein-kinin systems

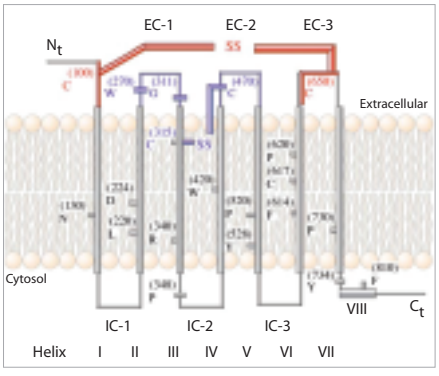


Structures of TOAC and POAC spin probes

We have proposed a broader and integrated line of investigation consisting of ten sub-projects, primarily based on the renin-angiotensin and kallikrein-kinin physiological systems. Peptides such as angiotensin, bradykinin and others participating in these systems, in conjunction with the corresponding analogues bearing unnatural structural modifications, will be examined. The receptors involved in these systems will also be evaluated through different approaches ranging from site-directed mutagenesis to synthesis, purification and conformational features of some of their hydrophobic transmembrane fragments. In addition to the classical structure-function approach, others such as those that evaluate the effect of radiation and proteolytic enzymes, upon selected peptide sequences, as well as others that explore the possibility of the involvement of certain peptides in tumor processes, will be examined.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The renin-angiotensin and kallikrein-kinin systems (RAS and KKS, respectively) have been investigated by our group through different approaches. A pioneering combined strategy involving the microscopic measurement of solvated peptide-resin beads and its electron paramagnetic resonance spectroscopy bearing amino acid-type spin labels, has succeeded in revealing relevant aspects of the dynamics of the polymeric matrix during the peptide chain growth. In this respect, the use of the spin probes TOAC (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid) and POAC (2,2,5,5-tetramethylpirrolidine-1-oxyl-3-amino-4-carboxylic acid), introduced in the peptides chemistry, have been very fruitful for addressing this issue. Moreover, these paramagnetic markers have been extensively applied for the development of several types of structure-function studies (in solution or in membrane-mimetic systems), mainly by handling labeled analogues of angiotensin II (AngII) and bradykinin (BK), the two most relevant physiological peptides involved in the RAS and KKS systems, respectively.



Schematic representation of the AGPCR 7-transmembrane (7TM) bundle structure, adjacent loops, and domains. TM helices I-VII and cytosolic helix VIII are indicated; ECs and ICs are extracellular and cytosolic loops, respectively. Segments of sequences consensual to all AGPCRs are shown in black; EC-1 and EC-2 loops found in the large majority of nonolfactory AGPCRs are shown in blue; insertions, including a second extracellular disulfide bond found in ANG II, bradykinin, endothelin, and other receptors, are shown in red. Amino acid residues are represented by the one letter code, and conserved positions used as references for alignment are indicated with the respective general numbers in parentheses. Oliveira et. al., Physiol. Rev. *2007.* ***87****:565-592*

Besides these approaches, the AngII and BK receptors (AT_1, AT_2 and B_1 and B_2, respectively) have also been targets for many investigations aiming at elucidating their roles in the mechanism of signal transduction of respective agonists. Efforts encompassing the synthesis of their structure specific fragments, deduced from site-direct mutagenesis and chimera expressions, have been carried out for further structure-functional studies. More recently, evidence of the binding site of the AT_1 receptor was obtained through EPR spectra, using active and inactive AngII analogues bearing TOAC spin probe in their structure. Significant intermolecular interaction of the active analogue ($TOAC^1$-AngII) was only observed with an AT_1 segment formed by the cyclization (Cys^{18}-Cys^{274}) of the N-terminal (13-17) and third extracellular loop (266-278) portions of the receptor and previously reported to be present in its binding region.

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BIOLOGICAL ASPECTS OF THIOLS: PROTEIN STRUCTURE, ANTIOXIDANT DEFENSE, CELL SIGNALING AND REDOX STATES

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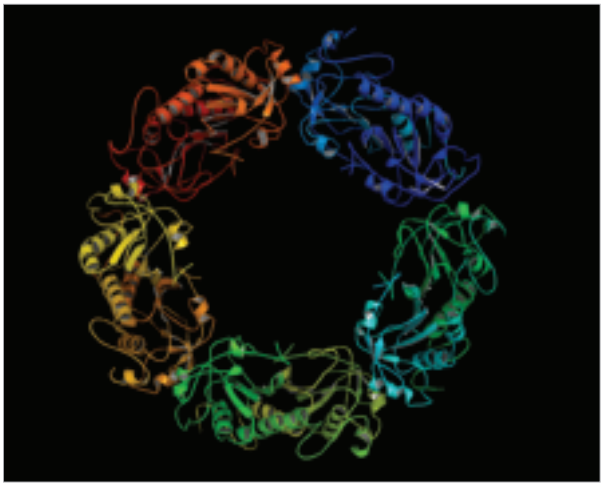

Figure 1. Tsa1 decameric crystal structure. Crystal of yeast Tsa1C47S was obtained by hanging-drop vapor diffusion in the presence of sodium citrate pH 4.2, 10 mM sodium chloride and 10% (w/v) PEG 3000 with 100 mM sodium fluoride as an additive. Each monomer is represented here by a different color. Figure was generated by the Pymol software (www.pymol.org). These results were published in Oliveira et al. *2007.*

The classical concept of oxidative stress is "a disturbance in the pro-oxidant-antioxidant balance in favor of the former". However, the accumulation of data has lead some authors to suggest that a more useful definition could be a "disruption of redox signaling and control". The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) plays a central role in the redox homeostasis, but there are also other thiols that participate in redox signaling. These thiols, which participate in redox pairs (RSH/RSSR), are enzymes that contain reactive cysteines (such as thioredoxins, glutaredoxins and peroxiredoxins), and are widely distributed. In contrast, most cysteine residues, both free and in proteins, possess low reactivity to undergo redox transitions. Appropriate protein folding in oxidoreductases generates environments in which cysteine residues are reactive. In this project, we propose to characterize both structurally and functionally several thiol systems, especially those derived from the model organism *Saccharomyces cerevisiae*. We have already elucidated the structures of several proteins that compose these systems and now we intend: (1) to elucidate novel structures; (2) continue to make functional-structural correlations and (3) determine the structure of protein complexes. Among these studies, we intend to further investigate a new antioxidant pathway: the reduction of 1-Cys peroxiredoxins by ascorbate (vitamin C). Our studies have changed the "thiol specific antioxidant paradigm" of these thiol-disulfide oxido-reductases and opened the perspective that ascorbate can interfere in the redox states of several thiol systems and, consequently, in redox signaling. We also intend to continue our characterization of antioxidant systems from *Xylella fastidiosa*. Previously, we elucidated the first structure of the organic hydroperoxide resistance protein (Ohr) from *Xylella fastidiosa*. Since Ohr is exclusively present in bacteria, this protein may represent a promising target for drug development. Unique structural and functional features of Ohr lead us to propose Ohr/OsmC as a novel family of antioxidant proteins. Other antioxidant systems from *Xylella fastidiosa* are also being currently analyzed.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

We are studying the physiological roles of thiol-proteins by employing multiple approaches such as enzymatic assays, crystallographic studies and microbiological investigations, by using yeast as a model. In this regard, we have purchased a collection of thousand of strains, each one with a different gene deleted. Detailed analyses indicated that although some of these proteins are partially redundant, they do present specific roles. As an example, glutaredoxin 1 and glutaredoxin 2 share a high degree of amino acid sequence identity (64%), but the latter enzyme is fifteen times more active than the former with respect to the monothiol mechanism. Several potential features involved in this phenomenon were postulated through structural analyses and we are in the process of testing them by site-specific mutagenesis. On a more dramatic example, Tsa1 and Tsa2 share 86% of amino acid sequence identity and present significant differences in the pK_a values of their reactive cysteine. We are currently refining a decameric crystallographic structure of Tsa1 (fig. 1), and this might provide us with information on its functional divergence. We suspect that these structural-functional variations may be related to protein-protein interactions, therefore we are also pursuing the elucidation of the structures of protein complexes. Another significant achievement of this project is the finding that ascorbate (vitamin C) can support the peroxidase activity of 1-Cys peroxiredoxins, which represents a change in the thiol-specific antioxidant paradigm. Finally, it is important to mention that our group was responsible for the description of the first structure of the Organic Hydroperoxide Resistance Protein from *Xylella fastidiosa* (fig. 2). The detailed characterization of this enzyme revealed unique features and led us to propose Ohr/OsmC as a novel family of antioxidant proteins. Since Ohr/OsmC proteins are exclusively present in bacteria (most of them pathogenic), Ohr appears to be a promising target for drug therapy, and in this regard, we are in the process of searching for potential inhibitors.

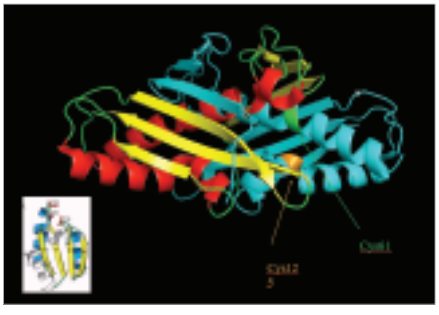

Figure 2. Ohr dimeric crystal structure. Crystal of Xylella fastidiosa *Ohr was obtained by hanging-drop vapor diffusion in the presence of 25% (w/v) PEG 4000 and 0.1 M Tris–HCl buffer (pH 8.7). One monomer is depicted in light blue while the other is in red, yellow and green. Cysteine residues involved in catalysis are shown in green and orange. Figure was generated by the Pymol software (www.pymol.org). These results were published in Oliveira* et al. *2006.*

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CELL PROLIFERATION CONTROL AND THE ORIGIN OF NEOPLASIA IN THE GENOMICS AND PROTEOMICS ERA

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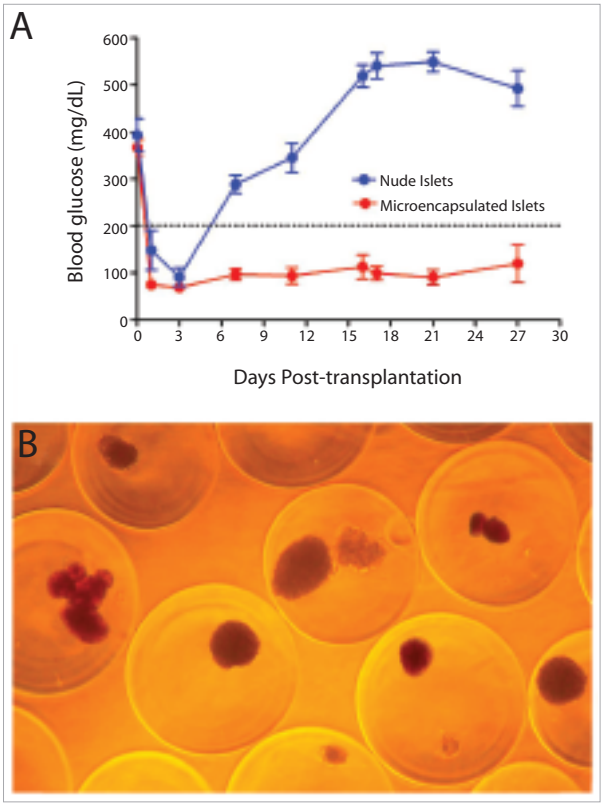


A: Reversion of diabetes induced by streptozotocin injection in immunocompetent Balb/c mice by microencapsulated human islets, shown in B

In the past two decades, the Cell and Molecular Biology Lab evolved to establish the Human Pancreatic Islet Unit and the Cell and Molecular Therapy Center (NUCEL – http://www.nucel.prp.usp.br), a center for translational research.

We focused mainly on cancer or the molecular basis for neoplasia, mainly, brain tumor (astrocytomas), prostate carcinoma, insulinoma and mammary carcinoma, by using state of the art technology to isolate and characterize genes which are differentially expressed in tumoral versus normal tissue. A number of collaborations were established with the medical community to allow access to patients samples, and new Bioinformatic tools were generated to deal with data obtained from using high throughput methods.

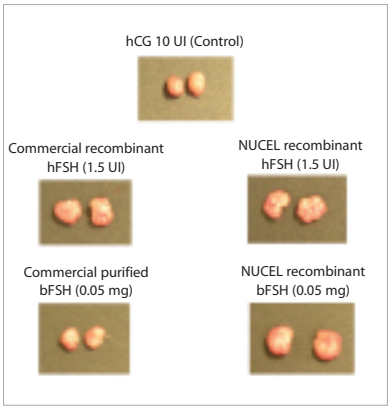
The group was also called upon to tackle another disease, namely, Diabetes mellitus (DM), which has been growing at a startling rate in the past few years, becoming a serious public health problem. Worlwide, around 10% of the almost 200 million diabetic patients are insulin-dependent (DM1), 5% of which are hyperlabile, with glycemic levels ranging from 50 to 2,000 mg/dL, which is seriously life-threatening. In 2002, our team introduced in Brazil an alternative treatment for these patients by means of pancreatic islet transplantation. However, since the islets are isolated from organ donors, this allotransplant requires immunosuppressive drugs, which cause secondary effects. We sought to establish a pre-clinical animal model for reversion of diabetes induced by streptozotocin, by using micro-encapsulated islets, and modifying the biomaterial used for encapsulation to improve islet survival and proliferation.

Organ shortage, relatively low islet yields and the need for a large number of insulin-producing cells to revert diabetes, prompted us to attempt to differentiate stem cells into insulin producing cells. Both murine and human embryonic and adult stem cells from different sources (umbilical cord, bone marrow, skin and dental pulp) are being used.

The ability to produce recombinant proteins allowed us to generate several products with great potential to become biopharmaceuticals, which are being transferred to the private sector for scaling up and commercialization.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Glioblastomas (GBM) are the most fatal tumors of the Central Nervous System, inefficiently treated with glucocorticoids (GC) chemotherapy. The quest for molecular markers for glioblastomas led us to use subtractive hybridization, DNA microarrays and Bioinformatics in a murine model system derived from the rat C6 glioma cell line, to isolate genes which are differentially expressed in ST1 and P7 rat C6 glioma variants, since the former is highly sensitive to GC while the latter is resistant to these hormones. DNA microarrays and Bioinformatics allowed us to identify a number of GBM markers, which may be used to generate DNA chips for diagnostics/ prognostics and new targets for gene therapy. New tumor markers for prostate carcinoma, insulinoma and mammary carcinoma were also identified.



In vivo *biological activity of human and bovine FSH (Follicle Stimulating Hormone) produced at the Cell and Molecular Therapy Center (NUCEL) compared to commercially available preparations*

We successfully established the animal model for diabetes and were able to cure this disease by injecting encapsulated human pancreatic islets into immunocompetent mice (Fig. 1). Cell Biology studies allowed us to propose new biomaterials for encapsulation of islets and other cell types. We were able to differentiate embryonic stem cells into insulin-producing, β-like cells, thus opening new avenues in diabetes treatment.

A number of cell lines overexpressing recombinant proteins were generated, which yielded products of biotechnological interest. For the first time in the literature, we obtained recombinant amylin/IAPP and its analogues, which are being used as insulin adjuvant to treat diabetes. Recombinant human and bovine FSH (follicle stimulating hormone), produced in high yields (Fig. 2), may be used for *in vitro* fertilization and animal management. Bone morphogenetic proteins (BMPs) 2 and 7, important inducers of bone regeneration, were produced and validated in pre-clinical trials. Secreted clotting factors VIII and IX were obtained and shown to display high biological activity *in vitro*. These and other products have attracted a number of biotech and pharmaceutical companies interested in transforming them in biopharmaceuticals.

In order to continue to pursue this translational research, the Cell and Molecular Therapy Center is forming new leaderships and building its own plant – a three-story building (1,600m^2) at the University Hospital area.

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STRUCTURAL AND FUNCTIONAL ASPECTS OF PROTEINASES AND INHIBITORS: CLONING, STRUCTURAL MODIFICATION, INFLAMMATION, HOMEOSTASIS, CELL BIOLOGY AND INSECT GROWING

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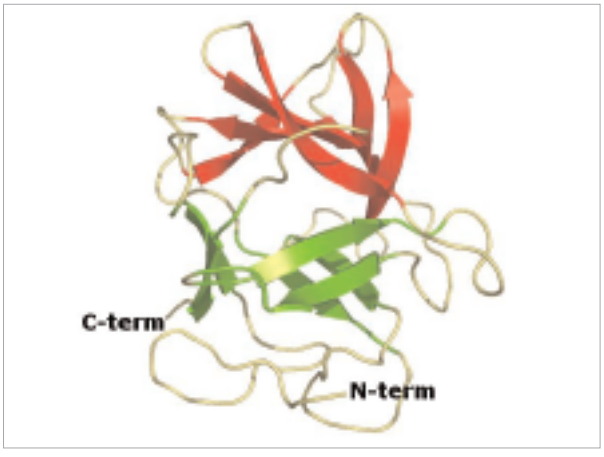
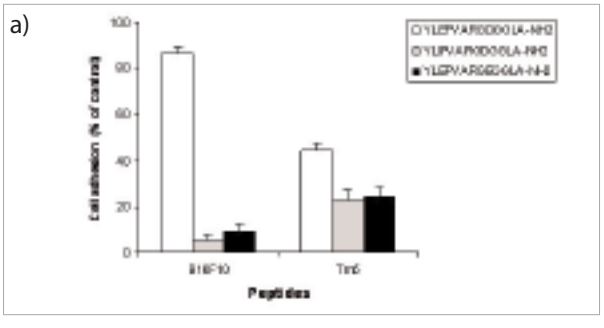
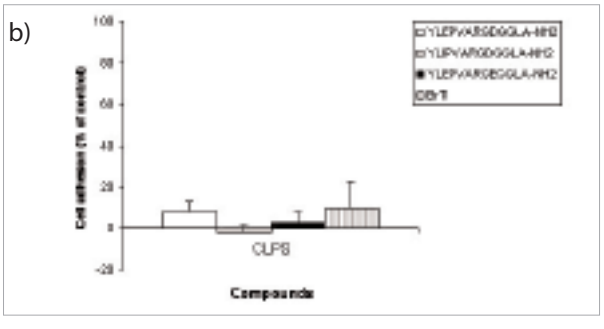


The overall structure of the recombinant Bauhinia bauhinioides *cruzipain inhibitor (rBbCI) . rBbCI has a , β-trefoil fold, formed by six two-stranded hairpins. Three of these hairpins form a barrel structure (in green) and the other three are in a triangular array that caps the barrel (in red).*





(a) Effect of synthetic peptides on cell adhesion of B16F10 (high-metastatic B16 murine mouse melanoma cell line) and of Tm5 (murine melanoma cell lines derived from a nontumorigenic lineage of pigmented murine melanocytes, melan-a). (b) The cell line was preincubated with synthetic peptides or BrTI (70 µM) for 15 minutes, and then subsequently added onto fibronectin coated plates (10 µg/ml) and incubated for 1 hour

Proteinases control a large number of key physiological processes such as cell-cycle progression, cell proliferation, cell death, DNA replication, tissue remodeling, homeostasis (coagulation), wound healing and the immune response. Also, these enzymes have been correlated with the invasion process of many parasites, demonstrating interactions with the host immune system.

Multiple molecular forms of inhibitor proteins have been characterized from microorganisms, animals and plants. These proteins are believed to participate in various physiological functions such as the regulation of proteolytic cascades and safe storage of proteins, as well as defense molecules against plant pest and pathogens. Concerning proteinase inhibitors, many members of the plant Kunitz family, purified from leguminous plants, showed an ability to inactivate some enzymes involved in blood clotting and fibrinolysis. Such inhibitors also exhibited anti-inflammatory effects, and decreased bradykinin release.

Brazilian forests, Pantanal and Cerrado regions are considered as great sources for natural products, among which are proteins capable of inhibiting proteinases. Such proteins can be used as models to study physiopathological mechanisms. Regarding clotting enzymes' inhibitors, the structural features that determine their specificity may be identified for target enzymes. For this purpose, the aim of this project includes: (a) the comparative study of serine proteinase inhibitors isolated from Leguminosae seeds; (b) primary and secondary structures determination, and modeling of inhibitors in order to identify specific amino acid residues important for biological action; (c) design of peptide substrates based on the inhibitor's reactive structure; (d) design of synthetic inhibitors structurally related to native proteins for blood clotting enzyme assays; (e) cloning and molecular modification for blood clotting enzyme assays will also be used on proteins isolated from Brazilian snakes, leeches and caterpillars; (f) characterization of the interaction between isolated and designed inhibitors and enzymes will be investigated in different biological processes such as inflammation, receptor and cell membrane association with endothelial cells, tumors, bacteria, yeast and the development of insect growth.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

We reported that the inhibitors BbKI and BbCI, obtained from seeds of *Bauhinia bauhinioides*, are 18 kDa proteins similar to other plant Kunitz-type inhibitors, but differ by the absence of disulfide bridges, and in their inhibition specificity. Following the heterologous expression and production of BbCI and BbKI recombinants in *E. coli*, both proteins showed potent inhibitory activities towards their respective proteinases, similar to the wild-type proteins. BbCI inhibits the human serine proteinase neutrophil elastase and pancreatic porcine elastase, and the cysteine proteinases cathepsin L and cruzipain from *Trypanosoma cruzi*. In spite of BbKI's structural identity to BbCI (84%), it differs from the latter by inhibiting plasma kallikrein, bovine trypsin and human plasmin. We are currently evaluating the inhibitory capacity of these proteinase inhibitors on cell viability of different tumor cell lines, primary human fibroblasts and on the proliferation capacity of human mesenchymal stem cells.

In parallel our collaborators also studied the interaction of human high molecular weight kininogen, a cystatin, with either endothelial or tumor cells. Human kininogens are intravascular proteins of blood plasma and play a role in cell and vascular biology. High molecular weight kininogen (HK) presents antithrombotic, antiadhesive and profibrinolytic activities. HK binds to endothelial cells where it can be cleaved by plasma and tissue kallikreins and release kinins. Heparan and chondroitin sulfate proteoglycans are described as kininogen receptors on the cell surface. This study analyzes the influence of proteoglycans on HK interaction with the cell surface. HK assembly, on endothelial cells (RAEC), was totally blocked by a peptide equivalent to a sequence from HK domain 5. Confocal microscopy experiments showed that HK co-localizes with heparan sulfate proteoglycans (HSPG) and cathepsin B on the cell surface. Our data show that HK is endocytosed by endothelial (RAEC) and tumorigenic cells (CHO-K1). In contrast, CHO-745 cells, which are almost completely devoid of glycosaminoglycan synthesis, do not take up HK. The endocytosed HK was detected in acidic endosomal vesicles. The process of HK internalization was blocked by low temperature, chloroquine, methyl-beta-cyclodextrin, FCCP and 2-deoxy-D-glucose indicating an active process of endocytosis dependent on membrane lipid raft domain. Cellular HK uptake occurs concomitantly with kinin release at the cell surface; besides serine protease, we also detected the involvement of neutral cysteine protease in kinin release. The present data report the HK endocytosis process by HSPG, as a novel additional mechanism for controlling kinin generation at the cell surface, suggesting that endocytosis of HK/HKa molecules is a control process of both angiogenic and antiangiogenic stimuli respectively mediated by these molecules.

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INSECT DIGESTION: MOLECULAR, CELLULAR, PHYSIOLOGICAL AND EVOLUTIONARY STUDIES

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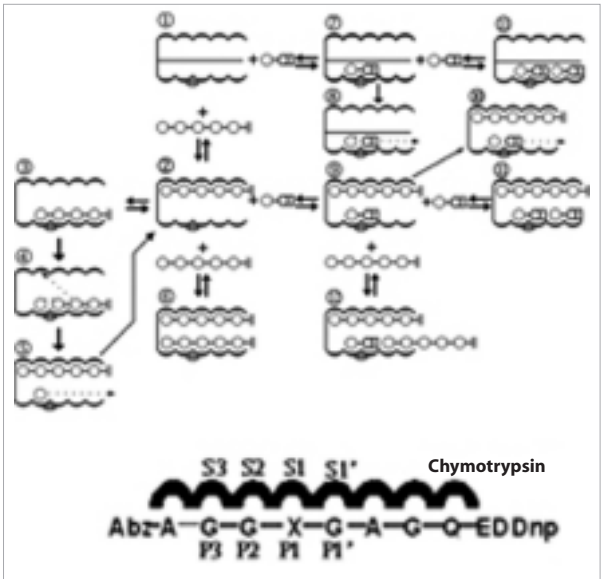


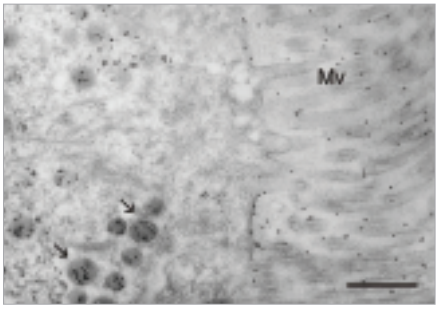
P. americana	0.6	0.1	Y	0.3
T. molitor	1.0	1.7	Y	0.5
D. saccharatis	1.8	0.2	Y	-1.5

Top: model for the action of a digestive enzyme from Abracris flavolineata. *Biochim.Biophys. Acta* ***1774****: 1079-1091 (2007). Bottom: insect chymotrypsin primary specificity (P1) and hydrophobicity (the figures) of their subsites. Insect Biochem. Molec. Biol.* ***38****: 626-633 (2008).*

Insects destroy about 10% of the word biomass each year and carry diseases to plants and animals, including man. However, the use of chemical pesticides to control insects causes environmental damages. The information that insect midgut is a large and relatively unprotected surface requires midgut studies to search for new targets of insect control. The insect midgut physiology needs a sophisticated understanding at the molecular level. It requires knowledge on digestive enzymes at the molecular structural level, as well as other proteins associated with midgut function like transporters, receptors, pumps and proteins participating in the enzyme secretory machinery. This project deals with these subjects in 4 main research lines: (a) digestive enzymology; (b) molecular midgut physiology; (c) midgut secretory enzyme machinery, and (d) evolution of digestive systems. Among our main goals is the study of structure and kinetics of digestive enzymes that led insects to overcome plant chemical defenses. The following enzymes will be studied within model insects: trypsin; chymotrypsin; cathepsins L and D; amino peptidase; trehalase; β-glucosidase; β-1,3-glucanase; α-mannosidase, and α-amylases. The development of midgut molecular physiology models of *D. peruvianus, T. molitor, M. domestica* and *D. saccharalis* will be based on several steps: (a) random sequencing of midgut cDNA libraries; (b) antibody for protein studies; (c) immunocytolocalization of recombinant proteins; (d) proposition of physiological roles for proteins, and (e) protein silencing by RNAi. Studies of insect digestive enzyme secretory mechanisms may result in valuable contributions to the physiology of digestion in insects and to cell biology. Screening midgut cDNA expression libraries with antibodies raised against proteins of microvilli cytoskeleton (*S. frugiperda*), cell apex (*T. molitor*) and microvillar membrane (both insects) will lead to the identification of positive clones. These will support secretory mechanism hypotheses following procedures including those described for the study of molecular midgut physiology.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

A study of the organization of digestion in the neglected major coleopteran group Dermestidae was performed to improve details on the evolution of digestive systems hypothesis. The major amino peptidase from the aphid *Acyrtosiphon pisum* was shown to be a lectin receptor such as a target for new control strategies. Chymotrypsins were purified from insects of 3 different orders and were compared regarding their substrate specificities with internally quenched fluorescent oligopeptides. They differ characteristically and the obtained data support aspects of a chymotrypsin catalysis mechanism. A glucanase purified from the grasshopper *Abracris flavolineata* and kinetically analyzed, indicated that the processivity results from consecutive transferences of substrate between accessory and active sites and that substrate inhibition occurs by the use of these sites. The cDNA coding for *Spodoptera frugiperda* midgut trehalase was cloned and expressed. Site mutagenesis of the recombinant trehalase identified the active site proton donor and nucleophile as D322 and E520, respectively. This is the first trehalase to have its active site groups identified by site mutagenesis. A recombinant cathepsinL-like (CAL) digestive enzyme from *Tenebrio molitor* was crystallized, submitted to X-ray diffraction and its 3D structure is being resolved.



Electron transmission immunocytochemical localization of PMAP in T. molitor *middle midgut. Arrows point to secretory vesicles. Bar= 0.5 µm.*

Antibodies raised against midgut microvillar proteins from *T. molitor* and *S. frugiperda* were used for screening cDNA expression libraries. The positive clones were sequenced, assembled and searched for similarities in databases. One of the predicted proteins from *T. molitor* was cloned, expressed and characterized. The obtained data support the assumption that this protein is comprised of domains A and B arranged in AB repeats. The processed form is AB and is putatively involved in peritrophic membrane formation. A predicted protein from *S. frugiperda* is annexin, and ongoing research suggests it is part of the digestive enzyme secretory machinery.

Glucose transport by *Dysdercus peruvianus* midgut cells were shown, *in vivo*, to depend on a facilitative transporter and a K+ -symporter. A cDNA coding for the facilitative transporter (GLUT) was sequenced and is being characterized.

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INTERACTION BETWEEN *Trypanosoma cruzi* AND HOST: LIGANDS, RECEPTORS AND DETERMINANTS OF INTRACELLULAR DEVELOPMENT

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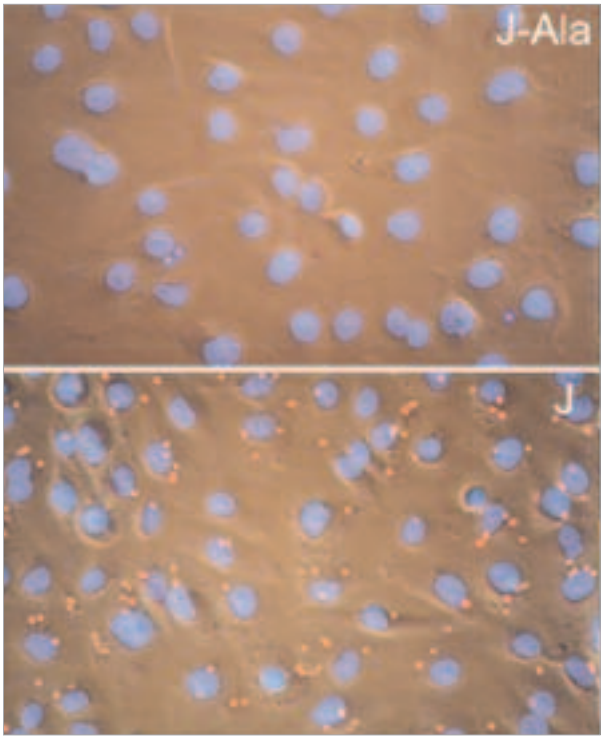


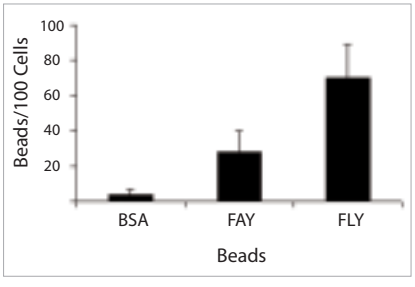
FLY binds to epithelial cell. Tissue-cultured cells were incubated with =(J). FLY-coated beads (red) or (J_Ala) FLA-coated beads (control peptide, = small blue spots). After washing, the cells were fixed with methanol and the nuclei were stained with Hoechst (blue)

Our Laboratory has dedicated in characterizing a family of proteins (TC-85) involved in the adhesion of the parasite to epithelial cells and to laminin. The objectives of our project are: (i) to verify if the adhesion domain of the TC85 family, selected *in vitro* for epithelial culture cells and components of the extracellular matrix, correspond to *in vivo* using phage display technique; (ii) to compare *in vitro*, with established lines, the results of adhesions of members of the TC85 family and their subfamilies in primary cultures of cardiomyocytes, neurons and Schwann cells, in order to verify if there is some correspondence between members of the TC85 family and different receptors of these different cell types; (iii) to identify, as a complementary procedure, molecules of the parasite which bind specifically to each component of the extracellular matrix. The same methodology will be used to identify, in the different cell types, the receptors for members of the TC85 family; (iv) to determine if the individuals of the *T. cruzi* population – intracellular trypomastigotes and amastigotes, express proteins of one or two principal subfamilies of TC85. For this, monoclonal (or monospecific policlonal) antibodies, aimed at exclusive epitopes of each subfamily, will be obtained and the parasites analyzed by immunofluorescence or high resolution microscopy; (v) to verify if members of the TC-85 family, or their peptides, induce changes in the host cell, such as increase in the synthesis of the extracellular matrix components or increase tissues and macrophages adhesion/endocytosis in culture cells; (vi) o study the transport of glucose and the expression of its transporter (mRNA and protein) and energetic metabolism throughout the intracellular differentiation of *T. cruzi* in the mammal. This data, compared to that referring to transport of proline, will make possible to establish the profile of the preferential sources of carbon in each stage. If this task proves successful, we will try to clone the transporters of proline and other aminoacids.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Chagas' disease is a chronic, debilitating and incapacitating illness, caused by the protozoan parasite *Trypanosoma cruzi* when infective trypomastigotes invade host cells. The main focus of our work is the identification and characterization of molecules involved in the interaction between the parasite and the host cell, as well as the transport of amino acids (proline, glutamate, aspartate) by the parasite, trying to understand the carbon and energy sources for the intracellular survival of the parasite.

Previously, we had described a glycoprotein family from *T. cruzi* involved in the adhesion step to the host cell (TC85). Recombinant proteins encoded by two genes of the family bind to laminin and to cytokeratin 18 and the binding sites were mapped. FLY, one of the conserved domains of the family is the cytokeratin binding site. FLY promotes dephosphorylation and reorganization of CK18 in epithelial cells and activation of the ERK1/2 signaling cascade culminating in an increase of approximately 9-fold in the number of parasites/cell. Inhibition of ERK1/2 phosphorylation blocks by 57% the host cell infection by *T. cruzi*. The possibility that FLY foracting as a pathogenic factor *in vivo* was then verified. Phages expressing the FLY domain bind specifically to the endothelium of organs affected in patients with Chagas´disease. Interestingly, FLY Balb/c mice, primed intraperitoneally with a single dose of FLY and infected with blood trypomastigotes, presented higher parasitism and mortality rates, with a decrease in IFN-gamma and NO production by spleen cells. The presence of FLY-containing molecules in "Membrane Vesicles", continuously shed from the surface of the parasite, may be the delivery system employed by *T. cruzi* to prime the host cell for infection. Membrane vesicles- treated animals develop severe heart pathology, with intense inflammatory response and higher mortality probably due to the increase of IL-4 and IL-10 synthesis.



Quantification of peptides binding to cells

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STRESS, TRANSPORT AND METABOLISM OF α-GLUCOSIDES IN *Saccharomyces cerevisiae*

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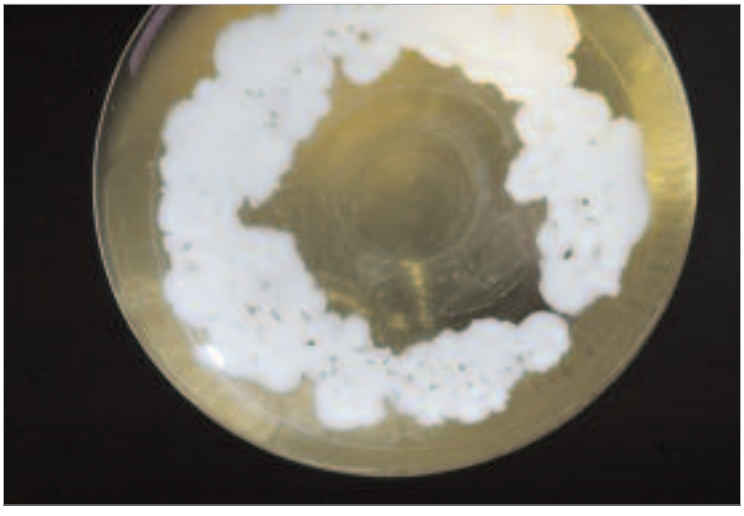


Colonies of S. cerevisiae

In this thematic project we intend to study cellular responses to stress conditions using the yeast *Saccharomyces cerevisiae* as a model organism for eukaryotic cells. We intend to explore the trehalose protective effects under stress conditions, focusing in oxidative stress. The tolerance acquisition mechanism under stress conditions that disrupt the cellular redox equilibrium will be our main interest in this research line. Since plasma membrane trehalose protection depends on the presence of the disaccharide on both leaflets of the membrane, the manipulation of the trehalose transporter transmembrane channel structure could lead us to dissociate the glucoside transport from the proton co-transport. Thus, the mutated gene could be expressed in other eukaryotic cell types, which would allow us to understand the conditions for dehydration resistance in those cell types. The study of the metabolic regulation for other α-glucosides besides trehalose could provide interesting results about the mechanism of his regulation, and eventually, led us to obtain strains that utilize these carbon sources more effectively. This could have some biotechnological interest. The mechanism of action of the purified and reconstituted into liposome's trehalose transporter will be studied both with the native as well as with the mutant proteins.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

A new metabolic pathway for the metabolism of α-methyl glucoside was described and characterized which involves the hydrolysis of the glucoside in the yeast cell periplasmic space.

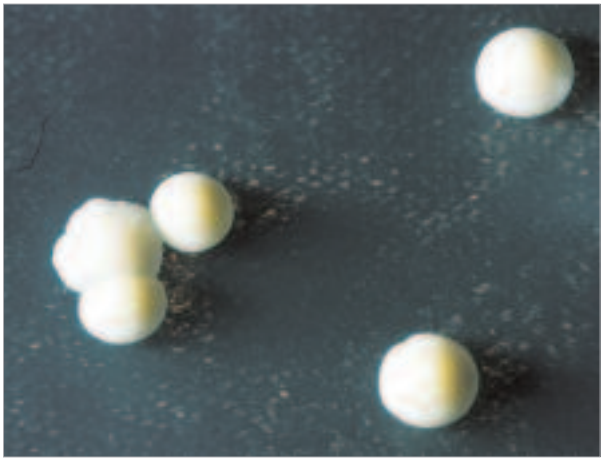
The negatively charged residues (Asp and Glu), which face the interior of the trehalose transporter transmembrane channel, have been replaced by alanines. In two mutant proteins, the glucoside transport became dissociated from the proton transport.

The asparagine residue from the same region was also mutated and this residue proved to be essential for the transport activity. An isoleucine, close to the asparagine, was mutated to threonine following an indication which appeared recently, that this substitution leads to an increase in the transport of maltotriose. However, no conclusive results were obtained in our case.

A construction linking gluthatione-S-transferase to the carboxyl end of the trehalose transporter was successful. This chimera is currently under study to obtain an efficient method for the purification of the trehalose permease.

The protective effect of trehalose was established for diverse stresses including those generating active oxygen species and cadmium yeast accumulation.

Saccharomyces cerevisiae *cells*



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MECHANISMS AND PATHOPHYSIOLOGICAL CONSEQUENCES OF REDOX PROCESS. EMPHASIS IN PROCESSES MEDIATED BY BICARBONATE BUFFER, SUPEROXIDE DISMUTASE1, THIOL PROTEINS AND NITROXIDES

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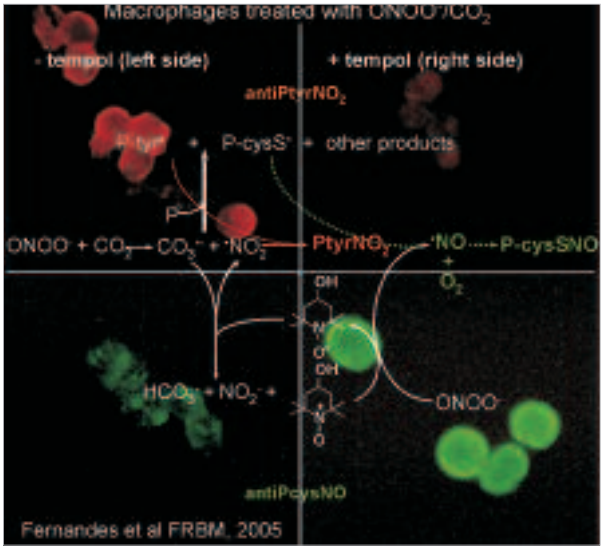

Diagram of the mechanism by which tempol nitroxide drives the reactivity of perixintrito/CO^2 from nitration to nitrosilation of proteins. (from Fernandes et al. 2005. Free Racic. Biol. Med. 38:189)

Currently, free radicals and oxidants are considered to mediate responses that range from signaling circuits involved in physiology and pathology to cellular and tissue injury. It is widely conceded that the elucidation of these many inter-related processes requires a better understanding of cellular oxidative mechanisms, including the identification of involved oxidants, the pathways regulating their generation and their targets at molecular level. In this context, we aim to continue to contribute to the elucidation of the molecular mechanisms and the pathophysiological consequences of redox processes by addressing questions that are timely and relevant to the human health.

Thus, the sources and fates of oxidants derived from bicarbonate buffer will be examined. We contributed to demonstrate that bicarbonate buffer modulates redox processes and are convinced that recognition of the oxidants derived from it will provide new perspectives to the understanding and control of numerous pathophysiological states and clinical conditions such as emhysema, respiratory muscle paralysis and pulmonary fibrosis. Also, we aim to advance in understanding structural and mechanistic aspects that modulate the pro-oxidant activities of the enzyme superoxide dismutase1. It is expected that these studies will contribute to the elucidation of neurodegenerative processes, in particular those associated with amyotrophic lateral sclerosis. In addition, we will continue to study the thiol proteins as controllers and sensors of biological oxidants because these proteins have only recently been recognized as players in redox processes and our previous kinetic and mechanistic studies provided insights about their multiple physiological roles. Finally, we will continue to study the mechanisms by which the nitroxide tempol is protective against oxidative and nitrosoactive conditions in vitro and in experimental animals. In our view these studies are relevant because nitroxides may constitute new antioxidant therapies, including to presently untreatable diseases such as multiple sclerosis and amyotrophic lateral sclerosis.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Our project aimed to contribute to the understanding of two general problems: (i) the biochemistry of oxidants derived from nitric oxide, such as peroxynitrite, nitrogen dioxide and carbonate radicals; and (ii) the mechanism and protection of tissue injury in inflammatory/infectious conditions by urate and nitroxides. I consider that the project developed well, although not all of the studies were finalized and published, in particular those involving animal models. In addition, as it is usual in scientific endeavors, some of the initial goals were redirected as a consequence of new insights obtained by us and other investigators during the project. Here, we report the main conclusions and perspectives of the project, limiting the references to our own work.

In regard to the biochemistry of nitric oxide-derived oxidants, we contributed particularly to the understanding of the physiological sources and fates of the carbonate radical. These studies led us to reveal other oxidants derived from the bicarbonate buffer, such as peroxymonocarbonate which was suggested to participate in the bicarbonate-dependent peroxidase activity of the enzyme superoxide dismutase1. We are convinced that recognition of oxidants derived from the main physiological buffer will provide new mechanistic insights into the understanding and control of numerous pathological processes and clinical conditions, such as emphysema, respiratory muscle paralysis and pulmonary fibrosis. Thus, these studies will be further pursued. In addition, we speculated that production of the carbonate radical during the peroxidase activity of superoxide dismutase1 promotes enzyme dimerization/aggregation as an important event leading to motor neuron degeneration in amyotrophic lateral sclerosis (ALS). In the next project, we aim to advance the understanding of the structural and mechanistic factors which modulate the pro-oxidant activities of superoxide dismutase1. In a previous study, we also advanced the comprehension of the mechanisms by which nitroxides inhibit tissue injury associated with conditions of oxidative and nitro-oxidative stress *in vitro* and *in vivo*. The potential of nitroxides as new therapeutic strategies makes it relevant to deepen these mechanistic studies and to examine tempol efficiency in protecting animal models animals of neurodegenerative processes, such as those associated with multiple sclerosis and amyotrophic lateral sclerosis. In parallel, it will be important to develop and test novel cyclic nitroxides as proposed in the current study. Finally, we should mention our recent studies which provided new perspectives to the understanding of peroxiredoxins as sensors and controllers of biological oxidants.

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REDOX AND CARBONYL STRESS ASSOCIATED WITH ENDOGENOUS ALFA-AMINOKETONES AND BETA-KETOACIDS: MECHANISMS AND BIOMARKERS

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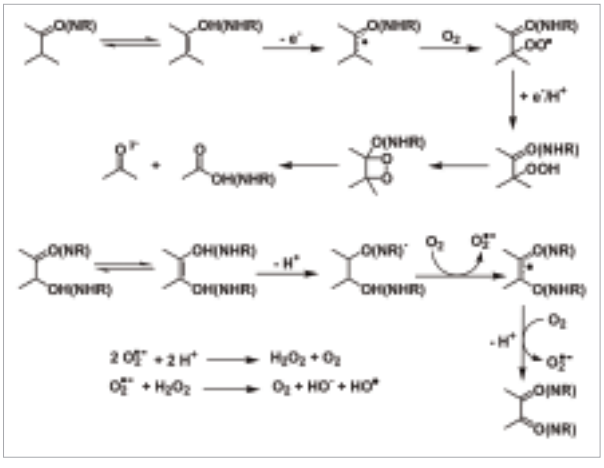

Radical acetylation of L-histidine and 2'-deoxyguanosine coupled to the reaction of peroxynitrite with diacetyl in aerated aqueous medium

Oxygen and nitric oxide are known to act normally as metabolic partners in biological events and any imbalance in this cooperative relationship results in oxidative and nitrosative damage to biomolecules and organelles, threatening the cell's life. Our research proposal focuses on the pro-oxidative and acylation properties of amino acid metabolites, particularly α-aminocarbonyls and β-ketoacids that accumulate in the tissues of individuals carrying metabolic errors. We aim to study: (i) 5-aminolevulinic acid (ALA), a heme precursor elevated in intermittent acute porphyria and lead poisoning; (ii) aminoacetone (AA), a threonine and glycine catabolite accumulated in diabetes, threoninemia, and *cri-du-chat*; (iv) succinylacetone, a β-diketone produced at high levels from tyrosine in tyrosinosis, where it leads indirectly to ALA overload; and (iv) 2- methylacetoacetate (MAA), an isoleucine catabolite abundant in isoleucinemia carriers. These metabolites undergo aerobic oxidation yielding cytotoxic and genotoxic species such as radicals, peroxides, triplet species and α-oxoaldehydes. The latter compounds encompass 4,5-dioxovaleric acid (DOVA), linked to ALA and SA accumulation, and methylglyoxal (MG), derived from AA and SA. In the next four years we plan to continue investigating the chemical mechanisms of ALA, AA, SA, and MA oxidation in aerated medium, triggered by metals, peroxynitrite, hemeprotein/H_2O_2 or hypochloride, as well as the damaging effects of their intermediates and final products – DOVA, MG, and biacetyl – on cell cultures, rats and humans. In parallel, we will developed analytical methods by CE/MS/MS for detecting these metabolites in biological samples and other biomarkers of carbonyl stress.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Aminoacetone (AA) was shown to induce apoptosis and necrosis in insulin-producing RINm5f cells that can be attributed to copper-catalyzed AA-generated superoxide radical, H_2O_2, and MG. AA-triggered cell death, as expected, is prevented by treatment with the antioxidant *N*-acetylcysteine (NAC). Flow cytometry studies indicated that the AA cytotoxicity to RINm5f implicates alterations in calcium fluxes, mitochondrial membrane polarization, and expression of BAX, BCL-2, and BCL-XL apoptotic proteins. Similar experiments with pancreas islets will be performed in an attempt to shed light on the role of those putative endogenous toxicants in diabetes. Regarding our work on ALA-related porphyrias (AIP, tyrosinosis, and plumbism), we have succeeded to establish a reliable experimental model based on i.p. injection of SA in rats, a strong ALA dehydratase inhibitor. Increased production of ALA, decrements in porphyrins, elevated indices of oxidative stress, and histological data are consistent with long reported mitochondrial changes observed in liver biopsies of AIP patients. In parallel, the hypothesis of lead-induced adolescent anti-social behavior is underway in Bauru (SP). Considering that ALA is an endogenous precursor of photosensitizing porphyrins, a by-product of our project is the formulation of a cream containing ALA methyl ester that was successfully used in photodynamic therapy of feline squamous cell carcinoma. Finally, our hypothesis on possible involvement of acyl radicals, generated by the reaction of α-dicarbonyls with peroxynitrite, in protein and DNA damage during nitrosative and carbonyl stress, seems indeed promising, once we verified authentic L-amino acids and 2′-deoxyguanosine acetylation by the diacetyl/peroxynitrite system. In the course of these investigations we have established CE/MSMS methodologies to analyze the metabolites mentioned here and glutathione-derived biomarkers of oxidative stress.

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PROTEIN SYNTHESIS IN EUKARYOTES

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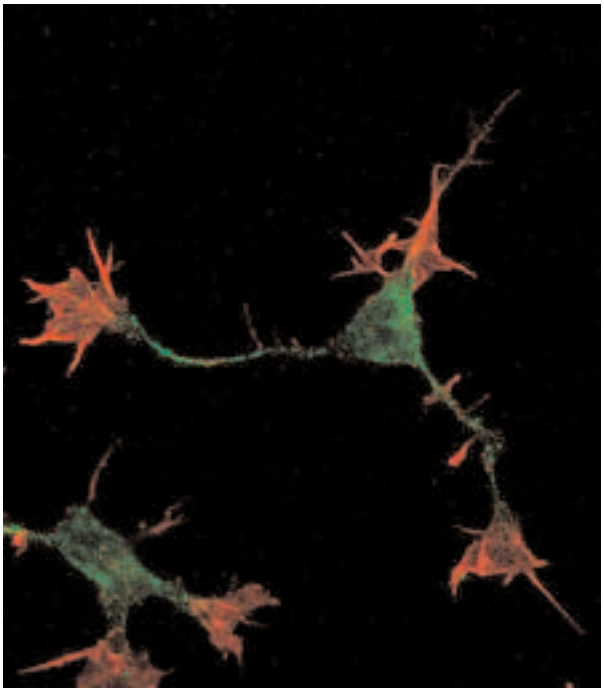

Distribution of IMPACT in neurons. Primary culture of hippocampal neurons (1 day in vitro*) stained with antibody anti-IMPACT (*green*) and rhodamine-phalloidin to label F-actin (*red*). IMPACT is found in the cell body and in neurites. Filamentous actin accumulates in growth cones, the leading tip of a neurite*

Protein synthesis in eukaryotes is regulated mainly through the activities of the initiation factors eIF2 and eIF4F. eIF2 is a heterotrimer, with the a subunit playing a regulatory role. Its phosphorylation by specific kinases results in inhibition of translation. The B subunit mediates interactions with other translation initiation components, and stabilizes the binding of GTP to the eIF2 complex; the y subunit, due to its similarity to EF-Tu, might bind GTP and the initiator tRNA. GCN2, the only eIF2a kinase found in the yeast *S. cerevisiae*, is activated by the binding of uncharged tRNA's. In mammals, besides GCN2, three other eIF2a kinases are known: PKR, HRI and PERK, activated by dsRNA, lack of hemin and endoplasmic reticulum stress, respectively. eIF4F, also a heterotrimeric factor, is responsible for the coupling of the 43S complex to the 5' end of mRNA ' s through the direct binding of its eIF4E subunit to the cap. eIF4E can be sequestered away from this interaction through its binding to 4E-BP, an interaction that is regulated by the phosphorylation of 4E-BP by mTOR. The activity of these factors, besides controlling general protein synthesis, can lead to the differential translation of rnRNA's, thus representing mechanisms for adjusting the synthesis of specific proteins to the immediate needs of cells, independently of transcriptional regulation. This project will address the function and regulation of eIF2 and eIF4E in *S. cerevisiae*, in trypanosomatids and mammals, focusing on the following specific aspects:

1. Functional study of the beta subunit of eIF2 in yeast – analysis of the association of eIF2B with eIF2y, and determination of the function of the C2C2 region of eIF2B.

2. GCN2-dependent translational regulation in yeast – determination of the function of proteins encoded by ORF's YDR152W and YLR419W, which share conserved sequences with the N-terminus of GCN2.

3. Translational regulation in trypanosomatids – studies on the phosphorylation of eIF2a, and characterization of eIF4E.

4. Translational regulation in the mammalian brain – mechanism of translation regulation in the experimental model of epilepsy; determination of the expression of GCN1 and GCN2 in brain; study of the function of the protein Impact, which shares conserved sequences with the N-terminus of GCN2.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Translational control mediated by phosphorylation of eI F2 plays a pivotal role in cell biology by inducing a downstream response aimed at cellular recovery from stresses. In mammals, four eIF2α kinases are found, GCN2, PKR, PERK and HRI, activated by different stress conditions. In the absence of this response, or when exacerbated, cell death ensues. Abnormally high levels of eIF2α(P) are found in several neuropathologies. In this project, we have shown that also in epilepsy there is a large increase in eIF2α(P) levels, caused by activation of PKR, leading to a drastic shut off of translation in the brain. On the other hand, this pathway is required for normal physiological responses in animals. GCN2 manages starvation for nutrients and determines feeding behavior and memory. During this project, we described a novel protein, called IMPACT, that functions as an inhibitor of GCN2. IMPACT was found predominantly in neurons, being extremely abundant in the hypothalamus. Our results suggest that under physiological stresses IMPACT functions to maintain constant protein synthesis required for neuronal signaling in brain areas that control homeostasis.

Translational control by eIF2 phosphorylation is also a means by which microorganisms adapt to changing environmental conditions. Trypanosomatid parasites, such as *T. cruzi*, *T. brucei* and *Leishmania*, encounter in their life cycles different environments in the mammalian host and insect vector. We have in this project addressed the eIF2 pathway in this group of parasites. We have characterized their unusual eIF2α subunit, and a unique trans-membrane kinase localized in the flagellar pocket in *T. brucei*, the only region of these cells with direct communication with the environment. These findings suggest that this novel eIF2 kinase is involved in cross-talks between host and parasite.

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CONTROL OF GENE EXPRESSION AND GENETIC PLASTICITY IN *Leishmania*

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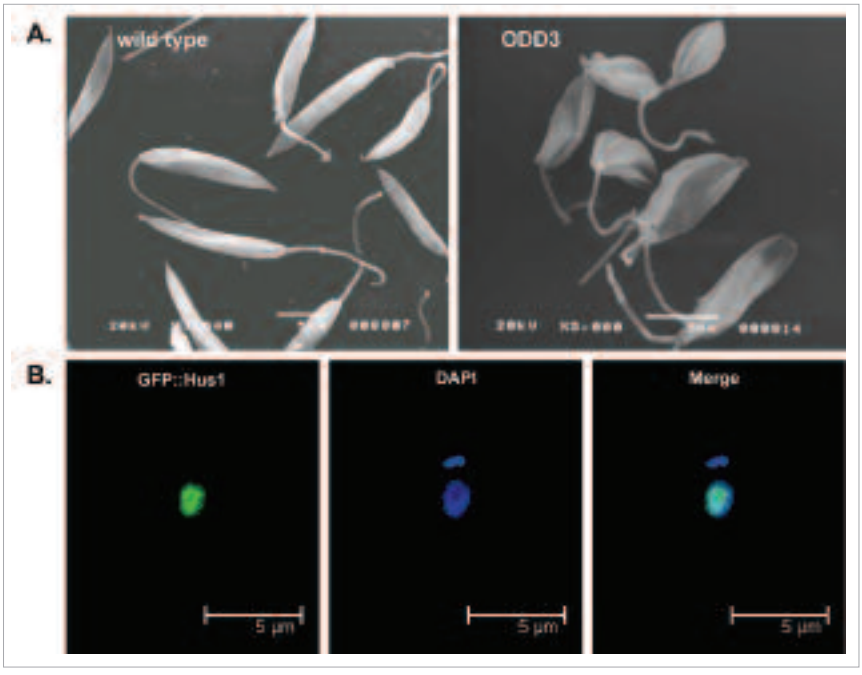

Different approaches are used to understand the control of gene expression in Leishmania

The proposal includes the studies of control of gene expression, genetic recombination and plasticity associated with comparative genomics of *Leishmania*. Different approaches and study objects are used to understand the control of gene expression in the parasite we are investigating: (i) noncoding RNAs as regulatory elements; (ii) a putative extraribosomal role of a ribosomal protein; (iii) the role of 5' and 3'UTR GKC to control its level of expression. Comparative genomics approaches. Parasite's genes possibly involved in DNA repair and recombination leading to genetic plasticity are also under investigation. Studies on aspects of genome organization affecting gene expression is being conducted by using bioinformatic tools followed by *in vivo* validation.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

We worked on the comparative analysis of three *Leishmania* genomes: *L. major*, *L. infantum* and *L. braziliensis*. This comparison revealed the presence of a few species-specific genes and a higher divergence of *L. braziliensis* genome and the presence of intact transposable elements and RNAi machinery only in this species1,2,3. We developed a pipeline to identify *in silico* conserved elements present in noncoding regions of the three *Leishmania* species. More than 70 conserved elements were found and we are currently investigating proteins which interact with two of these elements and their role.

Different routes used by the parasite to control gene expression are also under investigation; one of them is focused on noncoding SEQ CHAPTER \h \r 1RNAs (ncRNA). The overexpression of a putative ncRNA, the ODD3 gene, has been shown to impair growth rate of promastigotes in culture, to drastically alter the cell morphology and to diminish the level of a putative target transcript previously identified *in silico*. The co-transfection of this target transcript into *L. major* ODD3 overexpressors led to partial reversion of the original phenotype.

The investigation of mechanisms of genetic recombination and their role in the extensive genome plasticity of *Leishmania* is another theme of interest. In mammalian cells the protein Hus1 is involved in chromosome stability and DNA repair mechanisms. We found that the *L.major* LMHUS1 protein localizes to the nucleus and protects DNA from genotoxic stress. Our results also suggest the participation of LMHUS1 in cell cycle progression. We therefore hypothesize that LMHUS1 participates in chromosome stability and DNA rearrangements leading to DNA amplification. Another gene involved in DNA repair, control of replication, transcription and cell cycle progression is the Histone chaperone Anti-Silencing factor-1(ASF-1). We investigated its putative homologue from *L. major*, LmASF-1, and our results indicate functional conservation of the gene. Finally, the putative homologues of *Rad*51 and *BrcA*2, which are possibly involved in DNA repair mechanisms, are also being investigated.

While attempting to disrupt a telomere located essential gene of the parasite4 we have generated a heterozygous cell line bearing the selectable marker SAT (streptothricin acetyl transferase) integrated adjacent to the telomere of chromosome 20. The expression of SAT in this cell line was notably different when compared to a cell line in which the same selectable marker was integrated into an internal locus of chromosome 23. Notably, the pattern of SAT expression from this telomeric locus was affected by the increased expression of the LmASF-1 protein mentioned above. Altogether, our results suggested the possible existence of a telomere position effect that may participate in the control of *Leishmania* gene expression across telomeric loci.

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SINGLET MOLECULAR OXYGEN AND PEROXIDES IN CHEMICAL AND BIOLOGICAL SYSTEMS

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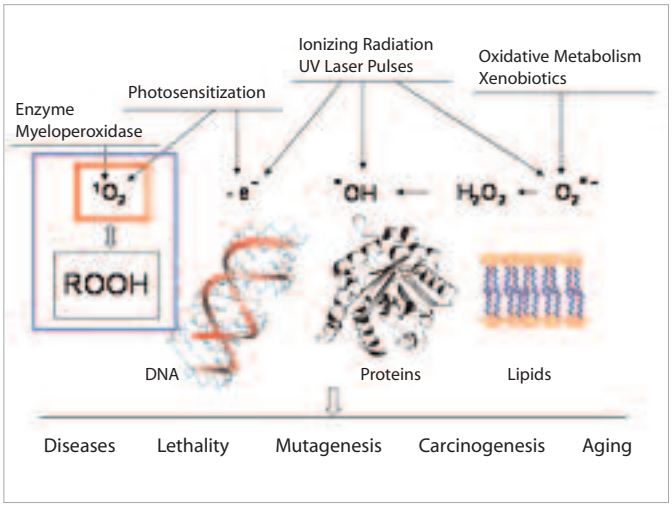

Singlet molecular oxygen ($^1O_2$) has been shown to be generated in biological systems and have been implicated in cell defense mechanisms against viruses and bacteria. Our studies focus on providing the mechanism by which $^1O_2$ and other reactive oxygen species play their physiological and pathological roles. We have been devoted to develop suitable $^1O_2$ generators based on the thermolysis of endoperoxides. These compounds are chemically inert and have been employed as versatile sources of $^1O_2$. This approach has been used in our studies for the detection of $^1O_2$-induced damage in cells (DNA, lipids and proteins) and for screening of biologically occurring compounds for quenching $^1O_2$.

Evidence has been accumulated during the last three decades on the strong implication of reactive oxygen species and one-electron oxidants in the generation of hydroperoxides from several nucleobases, amino acids and unsaturated lipid components. Singlet oxygen is also a major source of peroxidation of several key cellular components. The breakdown products of the rather unstable hydroperoxide (ROOH) precursors thus produced from exposure to endogenous or exogenous oxidizing agents may be implicated in deleterious biological effects such as cellular lethality, aging, mutagenesis and carcinogenesis. It may be added that oxidative processes to biomolecules are also involved in the etiology of other diseases including arteriosclerosis, arthritis, cataract and diabetes.

The purpose of the present project is to extend our understanding of the reactions between reactive oxygen species, specifically $^1O_2$ and ROOH with biomolecules *in vitro* and *in vivo* emphasizing the following aspects. Description of the main peroxidation reactions initiated by $^1O_2$ and ROOH within key cellular targets including pyrimidine and purine nucleobases, several lipid components and amino acids. Studies on the molecular effects of the initial formation of the above hydroperoxides within cellular components. Search of stable degradation products of biomolecules (ex. nucleobase) hydroperoxides that may be considered as the chemical signature of the formation of the latter unstable compounds that can be measured within cellular structure (ex. DNA, lipids, proteins). Indicators of lipid peroxidation that may involve cholesterol hydroperoxides and several degradation products including aldehydes. Measurement of adducts between amino substituted nucleobases and reactive compounds as aldehydes arise from the breakdown of initially generated unstable peroxides. Measurement of several side-chain altered amino residues that often arise from the fate of initially generated peroxides is used as bio-indicators of protein oxidation within cells.

Major efforts have been devoted to the elucidation of the mechanisms of peroxidation of major cellular biomolecules including nucleic acids, lipids and proteins. Relevant peroxidation pathways are now available at least for the main components of the key cellular biomolecules although there is still a need of further studies, particularly for isolating and characterizing putative hydroperoxides. Attempts should also be made to validate in the whole biomolecules the mechanisms of formation of hydroperoxides that were inferred from model studies. Another relevant major topic deals with the search of molecular signature of the peroxide/$^1O_2$ formation in targeted biomolecules within cells upon exposure to oxidative conditions. It may be anticipated that gentle and sensitive mass spectrometric methods such as tandem mass spectrometry (MS/MS) in association with HPLC and the use of ^{18}O-labeled peroxide/$^1O_2$ should constitute powerful tools for this purpose.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Our studies have focused on identifying the mechanism by which singlet oxygen ($^1O_2$) and other reactive oxygen/nitrogen species play their physiological and pathological roles. We have devoted efforts to develop suitable $^1O_2$ generators based on the thermolysis of endoperoxides. Few years ago, we synthesized the first water-soluble naphthalene endoperoxide isotopically labeled as a source of $^{18}[^1O_2]$. Synthesis and the use of the ^{18}O-labeled hydro peroxides derivatives (ROOH: lipids, proteins and nucleic acids) were also performed. The application of sensitive and specific methods, using mass spectrometry with electro spray ionization/MALDI-ToF developed in our laboratory, allows for the study of $^1O_2$/ROOH reaction in biological media, aiming to respond to the strong interest in the role of nutrition in the prevention and pathogenesis of cancer.

In our recent study, two cis and trans tryptophan hydro peroxide (WOOH) isomers were completely characterized by HPLC/mass spectrometry and NMR analyses as the major W-oxidation photoproducts. Using ^{18}O-labeled hydro peroxides ($W^{18}O^{18}OH$), it was possible to confirm the formation of a two-oxygen-labeled FMK molecule derived from $W^{18}O^{18}OH$ decomposition. In addition, these reactions are chemiluminescent (CL), indicating a dioxetane cleavage pathway. In summary, photo oxidation of W gives rise to a mixture of trans and cis hydro peroxides. Mechanistic aspects of WOOH decomposition were identified by different techniques: ^{18}O-isotopic labeling studies coupled to mass spectrometry analyses and light emission measurements.

Another interesting result, is the generation of 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde (ChAld) by reaction of cholesterol with $^1O_2$ produced by photosensitization or by thermo decomposition of 1,4-dimethylnaphtalene endoperoxide, a defined pure chemical source of $^1O_2$. On the basis of chemiluminescence's measurements and derivatization studies, we proposed that the mechanism of ChAld formation involves initial formation of a 1,2-dioxetane by $^1O_2$ attack at the 5¢ position. Our results add a new pathway that might explain the presence of significant amount of ChAld and 3β-hydroxy-5-oxo-5,6-secocholestan-6-al in neurodegenerative and cardiovascular diseases.

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STRUCTRAL AND FUNCTIONAL ANALYSIS OF MULTI-PROTEIN SYSTEMS INVOLVED IN THE PATHOGENICITY OF *Xanthomonas axonopodis* pv *citri*

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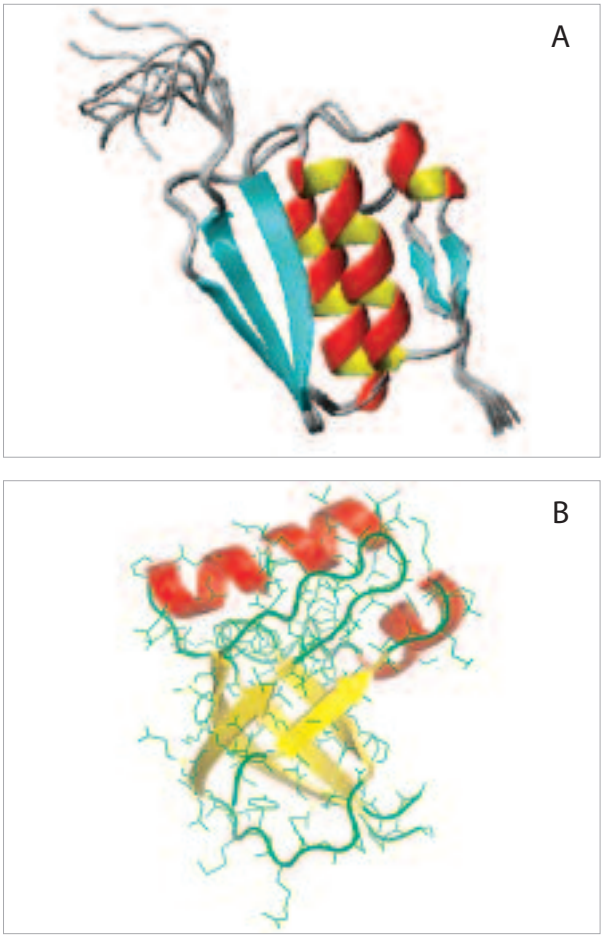

A) Solution structure of VirB7, a periplasmatic component of the Xanthomonas *T4SS. B) Crystal structure PILZ1133, of one of four PILZ domain-containing proteins coded by the* Xanthomonas *genome. PILZ1133 homologs are implicated in the control of bacterial motility. While some PilZ domains have been shown to bind c-diGMP, unique topological features in PILZ1133 structure abolish the nucleotide binding site*

In this project we propose to study some specific molecular systems that contribute to the pathogenicity of *Xanthomonas axonopodis* pv. citri *(Xac)*, the cause of citrus canker disease. We plan to use biochemical, spectroscopic and genetic techniques, in combination with the production of recombinant proteins to study the type III (T3SS) and type IV (T4SS) secretion systems and quorum sensing signal transduction pathway in *Xac*. We also plan to employ structural biology techniques to determine the structures of subunits and subunit complexes of the T3SS, T4SS and quorum sensing pathway. As well as providing details regarding the molecular mechanisms used by this phytopathogen to cause disease, these structures promise to reveal details regarding the function of orthologous systems found in other pathogens, including human pathogens with significant impact on public health.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Our current activities are focused on understanding the following systems:

i) Quorum sensing and c-diGMP signaling – Bacteria do not exist as solitary cells but instead coordinate their behaviors by means of extracellular chemical signals (autoinducers). We have recently shown that proteins involved in the detection of the *Xanthomonas* autoinducer DSF interact with proteins involved in intracellular signaling mediated by cyclic diGMP (c-diGMP), a second messenger implicated in the control of complex bacterial behaviors including motility, biofilm production and virulence. We are using biochemical and genetic approaches to characterize the molecular and physiological functions of several *Xac* proteins with roles in quorum sensing (RpfF, C, G) as well as proteins containing GGDEF, EAL, HD-GYP and PilZ domains involved in c-diGMP production, degradation and binding. We have recently determined the crystal structure of *Xac* PILZ1133 and are using NMR to study its interactions with other polypeptides.

ii) Type III secretion system – This multiprotein secretion complex works together with auxiliary cytosolic proteins to recognize virulence factors and transport them into the host cell. We are characterizing *Xac* strains with mutations in T3SS components and have demonstrated specific interactions between T3SS secretome components, cytosolic chaperones and secreted polypeptides. In one study we have shown that HrcU, an inner membrane T3SS component suffers autolysis and that the liberated fragment interacts with HrpB2 which is itself secreted. Another study is characterizing the involvement of specific *Xac* sigma factors in T3SS regulation.

iii) Type IV secretion system – We have identified a network of interactions among *Xac* T4SS cell envelope components and putative secreted factors. We are currently using NMR to resolve the solution NMR structure of the periplasmatic subunit VirB7 and study conformational transitions that occur upon its interactions with VirB9. The VirB7 structure revealed intriguing characteristics not observed in VirB7 proteins from other bacterial species.

iv) Structure and function of YAEQ – This new structure presents a variation of the PD-(D/E)XK motif encountered in a superfamily of metal-dependent nucleases that includes restriction enzymes and proteins involved in nucleic acid recombination and repair. YAEQ mutants have been produced and crystallized and *Xac* YAEQ knockout strains have been produced to study YAEQ function.

v) Structure and function of SUFE – We have determined the structure of *Xac* SUFE by molecular replacement. SUFE is a component of cysteine desulphurase in which it acts as an activator and transient receptor for elemental sulphur destined to be incorporated in Fe-S clusters of a variety of redox enzymes. We are currently characterizing its interactions with other components of this pathway.

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BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS OF *Leishmania* INFECTION

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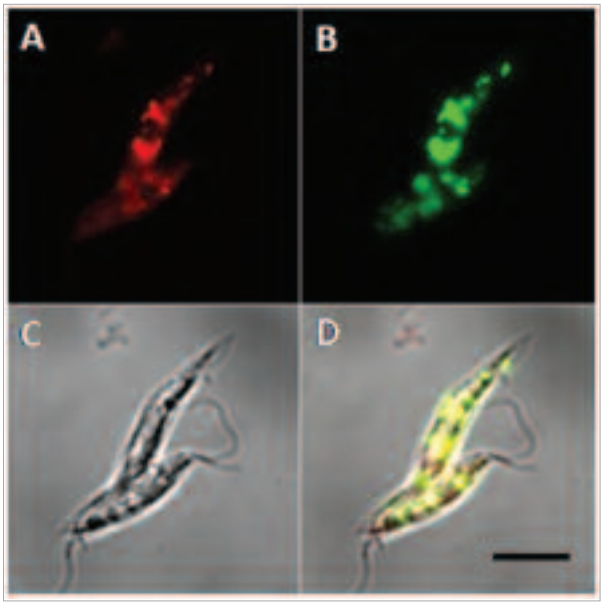

Confocal co-localization of glycosomal EGFP and arginase. Mutant Leishmania amazonensis *promastigotes expressing EGFPwith glycosomal signal were treated with anti-arginase polyclonal antibody. A. Image of arginase (red). B. Image of glycosomal EGFP (green). C. Phase contrast image. D. Merged image of glycosomal EGFP (green) and arginase (red). Bar = 5µm.*

Leishmania biology offers unique features that make it an attractive model to study pathogen-host interactions. The four sub-projects main goals are to study biochemical and physiological aspects of such interaction, mainly at mammalian host stage, where the parasite is infecting the macrophage.

The project intends to localize the sub-cellular compartment of *Leishmania* arginase. This localization will help in the elucidation of physiological role of the enzyme for its survival and maintenance of infection. We also want to determine if there is a competition for substrate between arginase and nitric oxide synthase induced in the macrophage.

The importance of the enzyme glucose-6-phosphate dehydrogenize (G6PD) and the pentose pathway will be evaluate in *in vitro* and *in vivo* infections to understand a possible role as a tool to detoxification of ROI and RNI. The transport of nutrients into the phagolysosome will be evaluated, as well as, the determination of the presence of few metabolic pathways, such as the ones to synthesize phosphatidyl serine.

To continue a previous study, the RNA polymerase I promoter region of *L. (V.) braziliensis* will be characterized and compared to other *Leishmania* sub-genus requirements for transcription of ribosomal RNA.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The main goal of the Project is to characterize enzymes presented in metabolic steps essential for parasite survival. The enzymes chosen are: arginase (ARG), glucose-6-phosphate dehydrogenase (G6PD) and phosphatidylserine synthetase (PSS). Besides, expression of ribosomal RNA is being studied by characterization of RNA polymerase I promoter organization.

Data of *Leishmania (L.) amazonensis* ARG coding gene was used to express the enzyme into *E.coli*. Protein purification yielded a high active recombinant enzyme and gave information about its structure. Polyclonal antibody raised against the purified enzyme allowed localizing its sub-cellular compartment in promastigotes expressing EGFP with glycosomal signalization.

Differences in G6PD coding sequence allowed the discrimination of *Leishmania (Viannia)* species. The target was then used in Real-Time PCR to quantify parasites in biopsies, a relevant information for epidemiological studies or in description of treatment protocols or even for applications of new chemotherapic tests.

We started the standardization of a protocol to quantify metacyclic forms in a promastigote culture (infective form), through real time PCR. The *Meta*1 RNA was used as target and the normalization was done with arginase RNA or G6PD RNA, known to be expressed all over the parasite life cycle.

The coding gene of PSS was obtained but the knockout was unsuccessful, an indicative that the gene is essential. Serine transportation from media to the cytoplasm was characterized with evidences of a transport system for the amino acid.

An IGS/ETS fragment encompassing the RNA pol I promoter region of *L. (Viannia) braziliensis* was cloned and is now being used to characterize the regulatory domains for rRNA transcription. In addition to RNA pol I characterization, we described that the acceptor site for *trans*-splicing can enhance reporter expression in heterologous expression vector, an important parameter to evaluate the cross recognition of the promoter region.

MAIN PUBLICATIONS

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FUNCTIONAL GENOMICS IN *Plasmodium*

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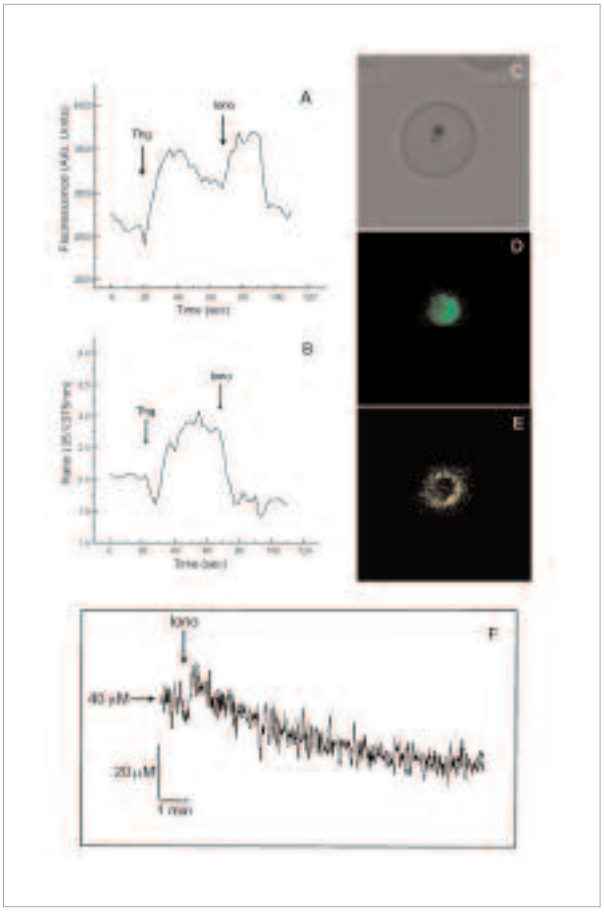

Simultaneous imaging of the [Ca^{2+}] in the PV and cytosol

The significance of this proposal is to investigate the adaptation of the parasite, where *Plasmodium* is capable of sensing the environment and using molecules derived from the host to signal the cell cycle. Using a bioinformatics approach we identified in the genome, four candidates with serpentine receptors in *Plasmodium falciparum*. Briefly, this proposal is based on the importance of the upstream signaling paths (serpentine receptors) and downstream paths (kinases, phosphatases, etc.) in *Plasmodium* for the modulation of transduction signal coupled to survival and replication mechanisms of the parasite. An important aspect of the project emerged with results of the microarray showing that *Pfemp* (*Var* genes) has its genes activated when parasites *P. falciparum* were subjected to treatment with melatonin. Investigation with strains of parasites coming from patient samples (obtained form the strain banks of the Department of Parasitology, ICB, Dr. Gerard Wunderlich, São Paulo and Rondônia) and comparison with culture samples treated or not with hormone could provide vital information for the understanding of the mechanisms of the regulation of the expression of the *Var* genes. One hypothesis is that the hormone could mimic a physiological situation and act in the regulation which is absent from the culture of *Plasmodium falciparum*. To unravel the paths of signal transduction from serpentine receptors in the membrane and target-proteins in the cytosol for the development of new drugs to combat malaria. To study the function of the genes, we plan to use bioinformatics, proteomics and systems Biology such as, for example, the use of synthetic genes with codon optimization to resolve the question of the difficulty of heterologous expression in *Plasmodium*.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

This thematic project commenced in March 2008, following on from an earlier thematic project, the objective of which was to elucidate the molecular and cellular bases of the transduction of the signal of the intra-erythrocyte cycle of *Plasmodium*.

Studies in Cell and Molecular Physiology in *Plasmodium* point to the use of a mechanism for transduction of signal which includes Ca^{2+}-ATPases and proteins regulated by calcium. The sequencing of the Genome of *Plasmodium* in 2002 revealed that 60 per cent of the genes of the parasite do not possess homology with other organisms. In this thematic project, we are investigating what mechanisms the malaria parasite would use to develop within the host cell, an inhospitable environment, using messenger seconds, such as calcium and AMPc to modulate its life cycle.

Our research shows that in the course of evolution, the parasite subverted the endocrine system of the host and sequestered calcium machinery creating an ionic microenvironment so as to adapt itself in the interior of the erythrocyte.

The passage between the intracellular stages, as well as the initiation of the cell division are synchronic processes. Interestingly the synchronicity of the intraerythrocyte forms observed in the cycle of *Plasmodium* is lost in culture. Our discovery that derivatives of tryptophan synchronize malaria parasite is the first evidence that the synchronicity depends on signaling molecules from the host. The hypothesis is based on the capacity of the parasite to perceive signal in the extracellular environment. The search for transduction paths of the hormone signal led us to find the participation of the protein kinase A (PKA).

In this project, we identified a receptor for kinase C *Pfrack* (Madeira et AL, 2003) in *Plasmodium*. This gene (*Pfrack*-GFP) when transfected in mammal cells, blocks the signaling of calcium when stimulated with the agonist ATP (photo attached).

The next question to be answered was: How could the parasite detect an extracellular signal? After a long search, we identified through bioinformatics 4 serpentine receptors in the membrane of *Plasmodium*. This information is fundamental for the understanding of how the parasite identifies and transduces extracellular signals.

Research into the mechanism of signal transduction by the malaria parasite represents a potential goal, as it could lead to the development of new chemotherapies.

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SUBSTRATES AND INHIBITORS FOR PROTEOLYTIC ENZYMES

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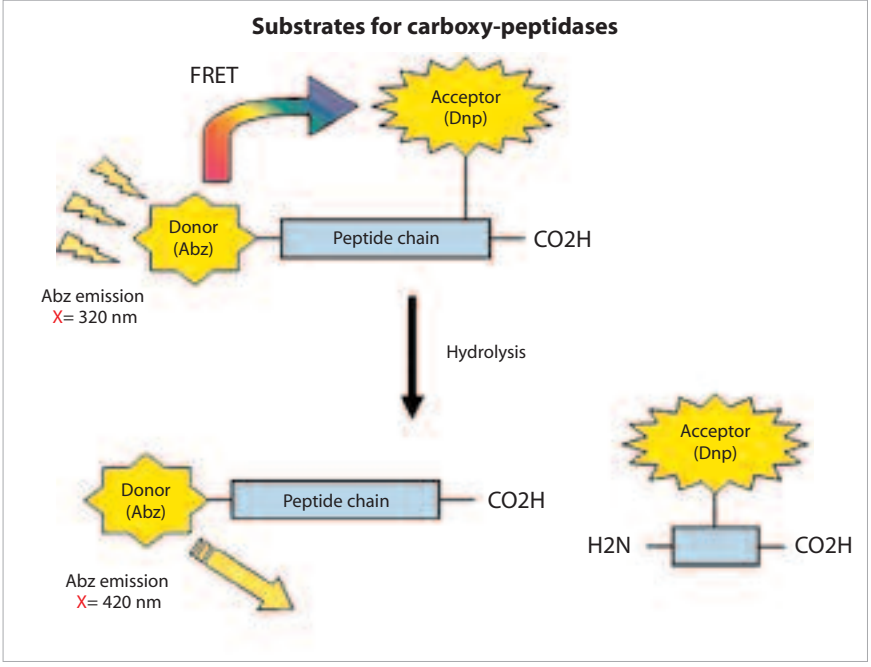
The purpose of this project is the development of organic synthesis of peptides and amino acids aimed at the study of proteases. We study a large diversity of proteolytic enzymes that have relationship with different physiological and physiopathological processes.

We have developed internally quenched fluorescent peptides and libraries, having as donnor-receptor fluorescent pair *ortho*-aminobenzoic acid (Abz) and N-[2,4-dinitrophenyl], respectively. The following proteolytic enzymes have focused in the last 5 years: a) Serine proteases:- plasma kallikreins, cathepsin G, human kallkrein 3 (PSA) and 6; b) Lisosomal cathepsin: F, K, H, S, V and X; c) Proteases from tropical parasite diseases as leishmania and malaria, and from virus as dengue, yellow fever; d) Endooligopeptidases:- Human prolyloligopeptidase, and oligopeptidase B from *Trypanosoma* e) Metalloproteases: angiotensin converting enzyme and PHEX, 24.11 and 24.15); f) Convertases (Kex2, PC1, PC2, PC5/6 and furin). Non-natural amino acids have been incorporated in peptides, and modifications on their peptide bond have been introduced in order to obtain specific substrates and inhibitors for the proteases we are studying.



Carmona AK, et al, Nature Protocols 1:1971 (2006)

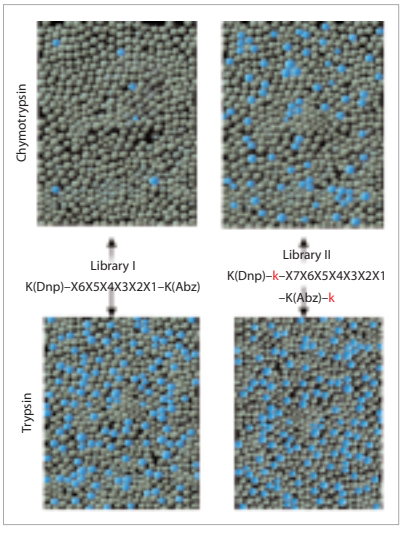
Our work is aimed also at the cell proteolysis, using the substrates and inhibitors that we are developing. In addition, we established a cell culture laboratory for these studies as well as to produce most of the proteases that we are proposing to study.

Physical-chemical measurements of some of the obtained peptides, as well as enzymatic activities have been done in order to correlate the form and function of substrates and inhibitors interacting with the proteases.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Continuous fluorescence resonance energy transfer for carboxypeptidases, application to angiotensin I-converting enzyme assay.

Angiotensin I-converting enzyme (ACE) is involved in various physiological and physiopathological conditions; therefore, the measurement of its catalytic activity may provide essential clinical information. This protocol describes a sensitive and rapid procedure for determination of ACE activity using fluorescence resonance energy transfer (FRET) substrates containing o-aminobenzoic acid (Abz) as the fluorescent group and 2,4-dinitrophenyl (Dnp) as the quencher acceptor. Hydrolysis of a peptide bond between the donor/acceptor pair generates fluorescence that can be detected continuously, allowing quantitative measurement of the enzyme activity. The FRET substrates provide a useful tool for kinetic studies and for ACE determination in biological fluids and crude tissue extracts. This methodology can be adapted for determinations using a 96-well fluorescence plate reader.



Beads of PEGA1900 resin from the libraries I and II after the incubation with trypsin (15h) and chymotrypsin (20 h) observed under the fluorescence microscope

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BIOENERGETIC, IONIC TRANSPORT AND REDOX EQUILIBRIUM IN MITOCHONDRIA

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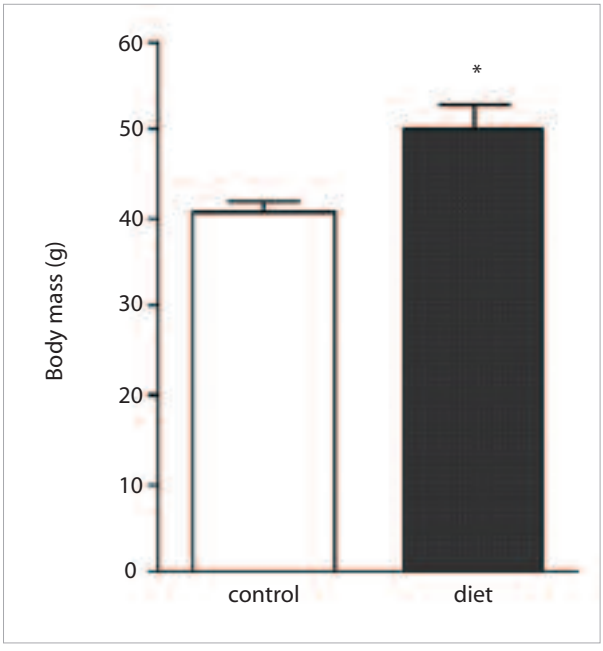

Hiperlipidic diet results in an increase in the body mass. A diet supplementation with vegetal oil during 12 weeks, had modified the swiss mice body mass

Changes in mitochondrial energy metabolism, ion transport and redox balance are associated with a variety of physiological and pathological conditions including aging, obesity, heart attack and stroke. The central aim of this project is to analyze the role of alterations in mitochondrial ion transport, energy metabolism and/or redox state in these physiopathological conditions. We expect, with our studies, to offer the possibilities for the creation of interventions capable of controlling these unwelcome mitochondrial and cellular effects.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Our main results are:

1) We were able to characterize clearly that the mitoK$_{ATP}$ channel is an endogenous mediator of the control of mitochondrial generation of species reactive to oxygen (EROs).

2) We were able to prove that light systemic and chronic decoupling in animals leads to a decrease in seric levels of glucose, triglycerides and insulin.

3) We began the establishment of a model system of caloric restriction incubating primary cultures of cerebral neurons in diminished quantities of glucose.

4) We carried out parallel measurements of oxygen consumption and generation of H_2O_2 in isolated mitochondria of brain (RBM), heart (RHM), liver (RLM) and kidney (RKM), in the presence of different substrates (α-ketoglutarate, glutamate, glycerol-phosphate, palmitate and succinate) and respiratory modulators (rotenone, antimycin, mixotiazol, CCCP).

5) We determined in this period that the model of caloric restriction in yeasts depends on the respiratory repression associated with high concentrations of glucose.

6) To determine mechanisms by which ischemic cardioprotection occurs, we verified that the NS-1619, putative activator of mitoK$_{Ca}$ channels, has several mitochondrial effects non-attributable to an activation of channels for K^+.

7) We verified that the pre-conditioning does not promote a large difference in the expression or localization of proteins of the Bcl-2 family, but that, after the reperfusion, pre-conditioned hearts present a larger quantity of antiapoptotic proteins Bcl-2 and Bcl-xL in their mitochondria.

8) Studies of the mitochondrial effects of cardiac post-conditioning still were not initiated in our laboratory, but should constitute objectives of future projects.

9) We verified that cerebral channels mitoK$_{ATP}$ are sensitive to redox state, and that they protect against excitotoxic neuronal death by preventing the cellular oxidative stress associated with this process.

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Schistosoma mansoni FUNCTIONAL GENOMICS APPLIED TO THE DEVELOPMENT OF VACCINES

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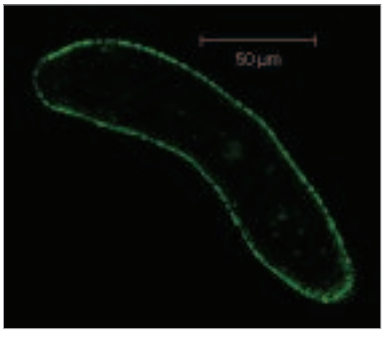
The *Schistosoma mansoni* EST Genome Project, in São Paulo, Brazil, has recently generated 163,000 ORESTES (open reading frame-expressed sequence tags) from 6 stages of the parasite, resulting in 31,000 assembled gene fragments. It is estimated that this dataset represents 92% of a total 14,000 genes of the parasite, increasing 10-fold the number of ESTs available and increasing to 522 the number of complete genes of the parasite. Automatic annotation based on attributions within the Gene Ontology system, provided a vision of important biological functions of the parasite and permitted identification of a set of proteins with characteristics which could indicate some potential as vaccine candidates. The gene/ proteins were selected based on their attributed functions, which may indicate a potential role in the survival of the parasite in the host and possible exposure for interaction with the immune system. Of special interest were proteins with functions such as toxins, surface receptors for cellular adhesion, surface proteins and ezymes, and receptors for host factors. Expression of the genes in the stages interacting with the host was evaluated. Furthermore, the transcriptome of the parasite revealed that ~55% of the sequences coded for protein with no definable function. Part of these may be associated with adaptation to parasitism; within these, genes which have increased expression in transition between cercaria and schistosomula/ adult worms, could have interest as vaccine candidates. In parallel to the Transcriptome sequencing, we carried out a pilot project for characterization of the potential protective effect of some possible antigens identified in the beginning of the project, using DNA vaccines for presentation of the antigens to the immune system. This pilot project permitted establishing the basis of a Functional Genomics project for characterization of the protective potential of the identified genes. The present project proposes to investigate a selection of genes/proteins as potential vaccine candidates, presented as DNA vaccines. It will be organized in 3 subprojects with the following objectives: 1) construction of DNA vaccines containing 25 candidate genes, characterization of the immune response induced against the respective antigens and evaluation of the protection induced against challenge with live cercaria; 2) characterization of the differential expression of the genes in the different life stages of the parasite by microarray using a panel of 4,000 selected genes; differential expression in parasites submitted to sera or cells from the immune system of immunized mice; 3) immunolocalization of the vaccine candidates in histological cuts of the parasite in the intra-host stages. This project should provide a first evaluation of the protective potential of a set of selected genes/proteins as vaccine candidates.

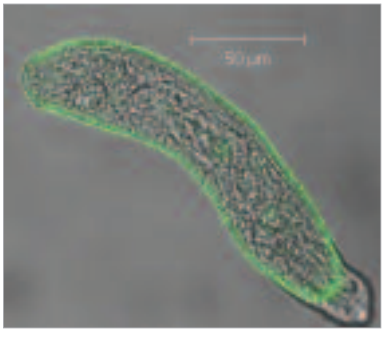
Immunlocalization of Dife5 Schisto 3h

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The São Paulo *Schistosoma* Genome Project generated an enormous amount of data from several stages of the parasite transcriptome. The data bank was used for an *in silico* identification of potential vaccine candidates, generating a list of genes/proteins with characteristics that could suggest their importance in the survival of the parasite, expression in intra-host stages and surface localization, which would allow interaction with the host immune system.

A preliminary screening evaluated the protection induced by DNA vaccines containing 30 of these genes, revealing a potential for 6 of these: a secreted exotoxin, Antigen 5, receptors for host factors, rVLDL and Stomatin, a surface enzyme, Apyrase, and 2 genes differentially expressed in the schistosomula stage. These antigens were submitted for patenting. Other antigens were selected from proteomic data.





Immunlocalization of Dife5
Schisto 7 day

Recombinant proteins were obtained for ~15 genes until now, allowing the investigation of different antigen presentations and the use of adjuvants, which may modulate the immune response induced towards conditions more favorable for the reduction of the parasitemia. The respective antibodies generated are being used to perform immunolocalization studies, confirming the surface localization of the antigens and their expression in intra-host stages. Furthermore, *microarrays* have been constructed: one with 4,000 gene fragments and the other with 44,000 oligonucleotides, representative of the parasite transcriptome. Parasites recovered from animals immunized with some of these antigens and protected against challenge have been analyzed as to the differential expression in relation to parasites recovered from control animals. On a whole these studies will allow a better evaluation of the potential of these antigens as vaccine candidates.

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G PROTEIN COUPLED RECEPTORS AND CHEMOSENSATION

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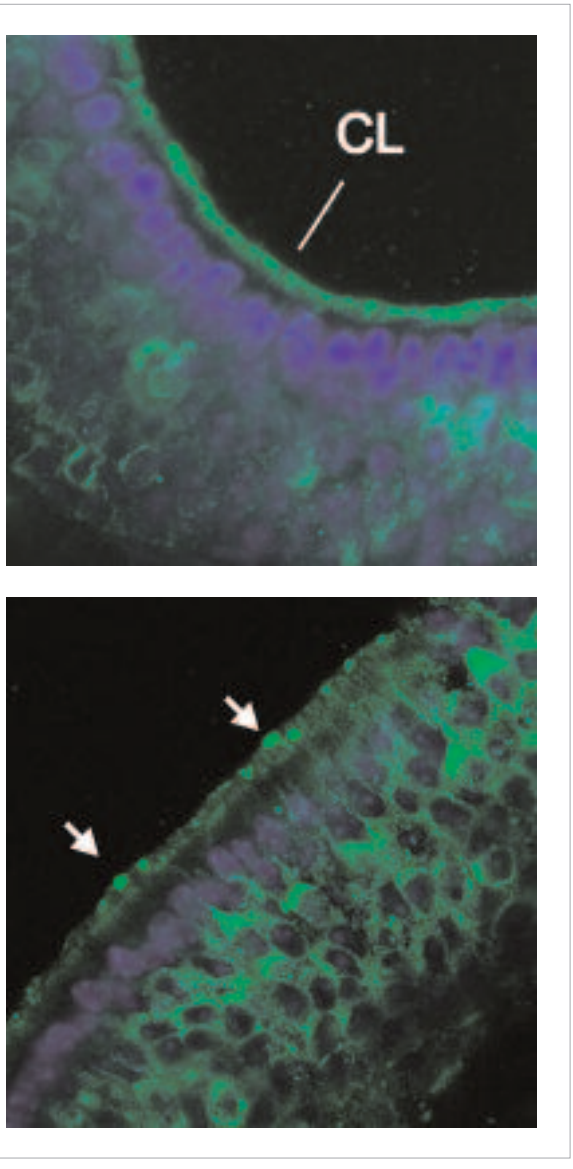

Immunofluorescence showing section through the olfactory epithelium of mouse using the anti-RIC-8B (green) antibody. The RIC-8B protein is located in the ciliar layer of the olfactory epithelium (CL) and also in the dendrites (arrows) of the olfactory nerve cells. Cell nuclei are colored with DAPI (blue)

Animals detect chemical stimuli present in the environment through a large number of receptors which belong to the superfamily of receptors coupled to the G protein (GPCRs). These receptors are expressed in different types of specialized cells, depending on their function. The olfactory receptors are expressed in the olfactory nerve cells, the taste receptors are expressed in the tongue taste buds, and the pheromone receptors, in the nerve cells of the vomeronasal organ. The activation of these receptors, by their ligands, releases a signal which results in the sensorial perception of the various stimuli. In the present project, we intend to analyze the following aspects related to two types of sensorial modality mediated by GPCRs, smell and taste: (1) how the transduction path of the signal of odorants in olfactory nerve cells is regulated *in vivo*. To this end, we intend to verify if the protein RIC-8B, recently identified in our laboratory, plays some role in this regulation. RIC-8B acts as a GEF (Exchange factor of GTP) on GαOLF, the olfactory protein Gα, which is responsible for the signal transduction through the olfactory receptors. (2) Mice present approximately 1000 OR genes, but just one OR gene is expressed in each of the olfactory nerve cells. We intend to investigate the mechanisms that control the regulation of the expression of the OR genes in the olfactory nerve cells. (3) Sweet tastes are detected by a heterodimer of GPCRs, the T1R2 and the T1R3. We intend to isolate molecules that modulate the function of human sweet receptors.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Odorants interact specifically with the olfactory receptors that are present in the cilia of the olfactory nerve cells. This interaction leads to the activation of the G olfactory protein, called GαOLF. We identified a protein called RIC-8B, which is an exchange factor of GTP (GEF) capable of binding to GαOLF and amplifying its activity. In this project we intend to study the functional mechanisms of RIC-8B, as well as determining its role in vivo. To-date, we obtained results which indicate that RIC-8B, in addition to interacting with GαOLF, interacts also with another subunit of heterotrimeric G protein, Gγ13. Our results represent the first example of a GEF which interacts with two different subunits of a heterotrimeric G protein. We also demonstrated that the Gβ1 subunit, is the , subunit predominantly expressed in the olfactory nerve cells, and therefore probable partner of GαOLF and Gγ13. Using an antibody against RIC-8B, we determined that this protein is also found in the cilia of the olfactory nerve cells, together with Gγ13, GαOLF and Gβ1. We also showed that RIC-8B increases the localization of G protein subunits in the plasmatic membrane of the cell. The results obtained up to now suggest two possible functions for RIC-8B in the olfactory nerve cells: RIC-8B may function as an accessory factor, which assists in the assembly and sending of G olfactory protein to the cilia of the olfactory nerve cells, and/or RIC-8B may act as a GEF to amplify the transduction of the signal of odorants. As next step, we will generate knockout mice, using stem cells where the RIC-8B gene is broken. These mice will be evaluated for their olfactory capacity.

The human T1R2/T1R3 receptor expressed in taste buds of the tongue is responsible for the detection of sweet taste and can be activated by different types of sugars, including sweeteners. The receptor has been expressed already in HEK293T cells and is being used through the SELEX method for the selection of aptamers of RNA which are capable of binding specifically to the site of the sucrose receptor. The identification of regulators for this receptor could contribute to the design of sweeteners.

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THE ROLE OF CELLULAR PRION PROTEIN IN PHYSIOLOGICAL AND PATHOLOGICAL PROCESSES

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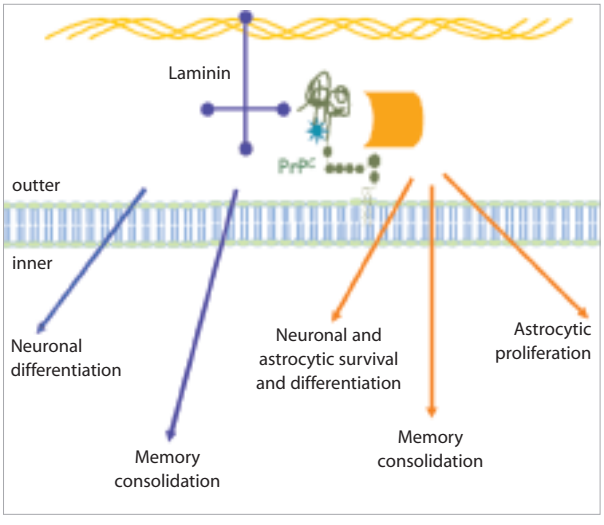

The Transmissible spongiform encephalopathies (TSE) or prion diseases are a group of fatal neurodegenerative disorders that affects both animals and humans and can exhibit sporadic, inherited or infectious presentations.

The propagation of the disease requires the expression of a GPI (glycosyl-phosphatidylinositol)-anchored cell surface sialoglycoprotein, the cellular prion protein (PrPC). This protein is converted into an abnormal form, called PrP^{Sc}, through a major conformational change. According to the protein-only hypothesis, the transmission of these diseases does not require nucleic acids, and PrP^{Sc} itself is the infectious prion pathogen.

Most research in the prion field is directed at understanding the nature of the infectious agent, and the mechanistic and structural aspects of the PrP^{C} conversion to PrP^{Sc} in either infectious or mutation-related pathologies. Nonetheless, the diagnostic procedures available for prion diseases are less sensitive than required and therapeutic interventions for these devastating diseases are still elusive. Furthermore, the risks related to iatrogenic contamination are significant, inclusive the use of blood components, because possible infection by blood transfusion has been recently described in two cases in UK.

The accumulation of the toxic insoluble PrP^{Sc} has been taken as the most probable event responsible for neuronal death in prion diseases. However, since clinical manifestations may occur either before or without characteristic PrP^{Sc} deposits, it has been suggested that neurotoxicity is unlikely to be the unique factor in the pathogenesis of such diseases.

In the last few years several biological functions of PrP^{C} have been uncovered. The expression of PrP^{C} is ubiquitous and its interaction with cellular partners mediates cellular survival, differentiation and proliferation. This large spectrum of PrP^{C} cellular functions indicates that PrP^{C} loss-of-function could be associated with the pathology of prion diseases but additionally, that alterations in PrP^{C} activity may also be related to other pathological processes.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Strong evidence for a neuroprotective PrP^C function derives from our description of a putative PrP^C p66 ligand, which was later identified as the Stress Inducible protein 1, STI-1. The interaction between PrP^C and STI-1 prevented programmed cell death and induced neuronal differentiation using cAMP-dependent protein kinase (PKA) and ERK1/2 signaling pathways, respectively. PrP^C also engages to vitronectin which promotes axonal growth in dorsal root ganglia (DRG) neurons. Important clues of PrPC functional properties were also provided by our observation that PrP^C is a cell surface ligand for laminin and mediates neuronal differentiation.

The normal functions of PrP^C -LN or PrP^C-STI1 were approached *in vivo* and we showed that the association between these molecules induces memory formation and consolidation. These results are particularly relevant for the loss-of-function hypothesis since cognition impairment is one of the initial signals of prion diseases.

PrP^C functions in astrocytes were also addressed and it was demonstrated that PrP^C-STI1 engagement inhibits astrocyte proliferation. Remarkably, astrocytes are able to secrete STI1 which works as a trophic factor for neurons and as an autocrine factor for astrocytes. In human glioblastoma cell lines, contrary to its effect in astrocytes, STI1 promotes PrP^C-dependent proliferation. These data point to an opposite role of PrP^C in normal versus tumoral cells, making PrP^C an interesting therapeutic target to control tumor growth.

All together, our discoveries point for diverse and important physiological functions of PrP^C and support the hypothesis that PrP^C loss-of-function can participate not only in some of the pathological signals of prion diseases but also in other human diseases such as cancer.

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DAMAGE TO BIOMOLECULES INDUCED BY REDOX SYSTEMS. MECHANISTIC STUDIES AND DEVELOPMENT OF SENSITIVE BIOMARKERS

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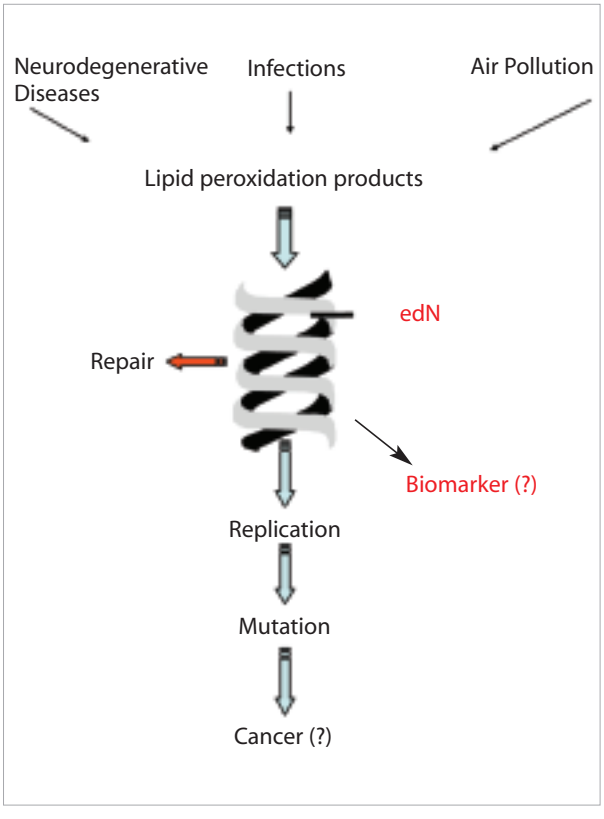


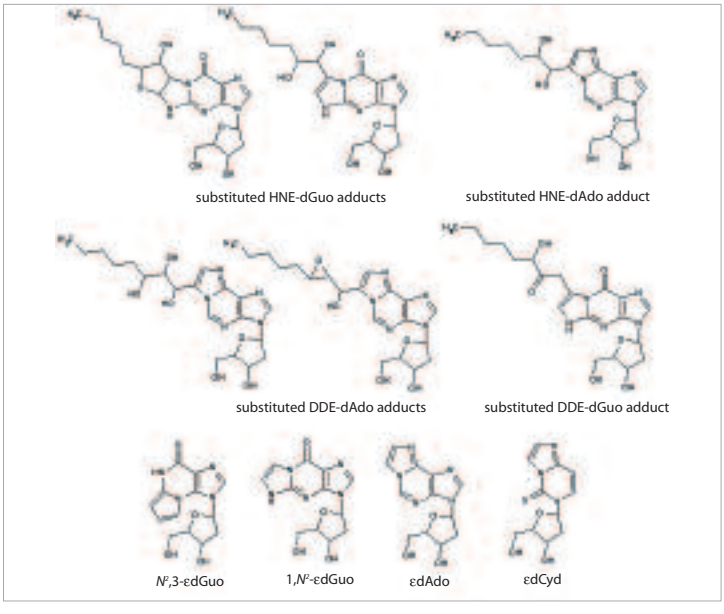
Models studied in this project leading to lipid peroxidation products which form exocyclic DNA-base damage

A very large body of studies has focused on the role of redox-dependent mechanisms in a broad variety of disease conditions including cancer. A basic and prime drawback that prevents a better understanding of redox pathophysiology is the overall difficulty and the almost lack of availability of adequate indexes to quantitatively assess the redox status of *in vivo* systems, including cells, tissues and whole organisms. Redox processes induce direct or indirect damage to biomolecules. It is well known that lipid peroxidation products as acrolein, crotonaldehyde, trans-2-hexenal, trans, trans-2,4-decadienal and, trans-4-hydroxy-2-nonenal can alkylate DNA and proteins producing adducts. The ability of such molecules, to react with biological targets, and exert cytotoxic effects, is only beginning to be studied. The aim of the present study is to investigate the mechanisms and the products of redox damage to biomolecules by ultra sensitive methods. The levels of these adducts will be detected and quantified *in vitro*, by test tubes and cell cultures, and in model systems, by transgenic rats with mutant SODI and rats exposed to urban air pollution. The possible mechanisms of adduct formation and the effect of antioxidants will be also investigated.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Our laboratory has focused on DNA and protein damage by aldehydic bifunctional electrophiles that covalently modify DNA and proteins. We reported that *trans,trans*-2,4-decadienal (DDE)-oxidation products can bind to adenine and guanine in DNA yielding adducts. In addition to 1,N^6-etheno-2'-deoxyadenosine (εdAdo) and 1,N^2-etheno-2'-deoxyguanosine (1,N^2-εdGuo), six novel etheno adducts were identified. An ultra-sensitive method involving on-line reverse-phase high performance liquid chromatography with electrospray tandem mass spectrometry detection was developed for the analysis of etheno adducts in DNA. The modification of cytochrome c promoted by DDE was also demonstrated by ESI-Q-TOF mass spectrometry analysis. Presently we are investigating the role of these adducts in amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease. The association between redox processes and ALS has been strengthened by the discovery that mutated super oxide dismutases are associated with the neurotoxicity. DNA and protein adducts appear to be promising markers in molecular studies to assess the protective effects of antioxidants against biomolecule damage involved in the development of the disease. Markers of redox state may allow the early detection of lesions in biomolecules and their causes, increasing significantly the maintenance of human health.

Structures of some ethano and etheno adducts formed upon reaction of oxidized α,β-unsaturated aldehydes with DNA bases



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FUNCTIONAL CHARACTERIZATION OF GENES POTENTIALLY REGULATED BY ESTROGEN RECEPTOR AND/OR *Erbb2* ONCOGENE: IMPLICATIONS IN THE DIAGNOSIS, PROGNOSIS AND TREATMENT OF BREAST CANCER

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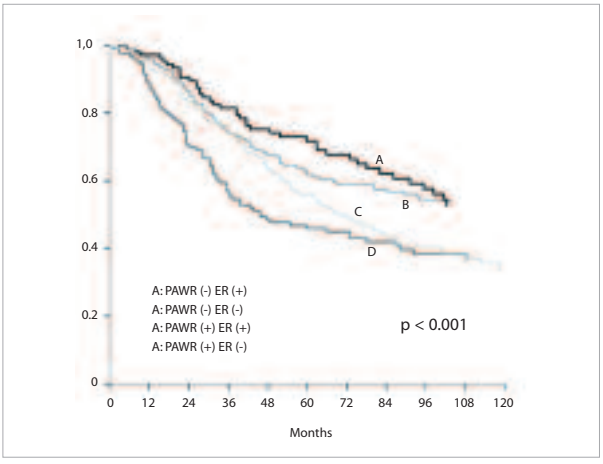
Breast cancer is the most commonly diagnosed neoplasm and the major source of morbidity and mortality among women worldwide. In Brazil, breast cancer is one of the most frequent malignancies in women and the leading cause of mortality. Data from the Ministry of Health estimates the occurrence of 49,400 new cases of breast cancer in 2008, representing an important health problem (INCA, Ministério da Saúde, 2008). Currently, the great deal of breast cancer research has been the identification of new diagnostic and prognostic factors for the disease and the understanding of critical signaling pathways involved in the carcinogenic process of the breast, which could provide the knowledge base for the identification of new therapeutic targets and new predictive markers of tumor sensitivity.

Using different techniques for gene expression analysis, such as DDRT-PCR, cDNA microarray, SAGE and Real Time PCR, in primary breast tumors regarding the presence or absence of ER and PR and two human mammary luminal cell lines expressing different levels of *Erbb2* before and after intensive exposure to docetaxel, we identified a large number of differentially expressed genes that could be considered as potential candidate markers for breast cancer. However, the functional role or clinical significance of these genes in breast cancer development and progression is still unknown.

The present study involves a group of qualified researchers with different expertise aiming to investigate the functional role and the prognostic value of the differentially expressed genes identified by our group, in breast cancer. To achieve these goals, six sub-projects will be performed. The first four are experimental studies involving the use of cell culture system in monolayers and in 3D, transient transfection with expression vectors, and suppression of gene expression by small interfering RNA (siRNA). In the fifth and sixth sub-projects, the Tissue Microarray technique (TMA) will be used to evaluate the clinical and prognostic significance of new candidate tumor markers in a large and well-characterized series of tumor samples and benign lesions of the breast. In addition, the expression of several panels of genes involved in different biological pathways will also be analyzed by IHC on TMAs.

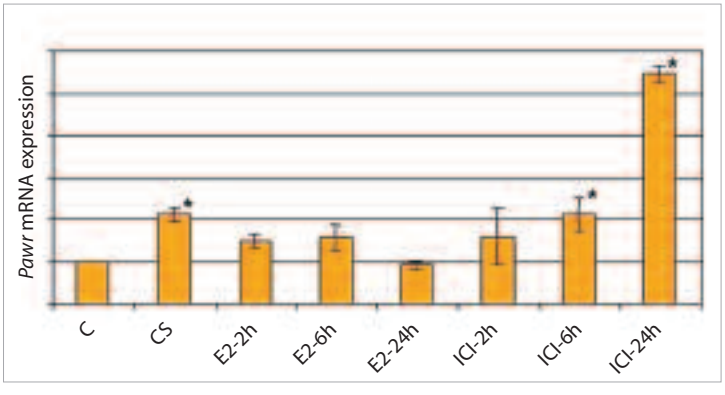


Kaplan-meier curves for long-term overall survival in breast cancer patients, stratified according to PAWR and ER protein expression. Tumors are classified in four categories according to the immunostaining status of PAWR and ER protein: PAWR-/ER-; PAWR-/ER+; PAWR+/ER-; PAWR+/ER+

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

To date we have preliminary results by real time RT-PCR confirming that several of the selected candidate genes are regulated by estrogen and/or growth factors, such as EGF (epidermal growth factor) in breast cancer cells, leading to the identification of a set of biomarkers candidates, whose expression in breast cancer cells, is selectively regulated by these cell signaling factors. We also showed that the transcripts of *Lrrc*49/*Thap*10 bidirectional gene pair are co-regulated by estrogen and that hypermethylation of the bidirectional promoter region simultaneously silences both genes in a subgroup of primary breast tumors. Moreover, among the genes evaluated in this study, the *Pawr* gene (*P*KC *a*poptosis *w*t1 *r*egulator gene; also named *Par*-4, *p*rostate *a*poptosis *r*esponse-4) that encodes a 38 KDa protein containing a death domain and a leucine zipper domain with pro-apoptotic activity, showed to be negatively modulated by estradiol and EGF in MCF-7 breast cancer cells. Our data using the pure antiestrogen ICI 182 780, also indicate that there might be a cross-talk between ER (estrogen receptor) and EGF pathways on the modulation of *Pawr*. In addition, immunohistochemical analysis of a wide panel of breast tumors was conducted to assess the prognostic value of the PAWR protein. By IHC on TMAs, fifty-two percent of the breast tumors showed positive PAWR protein expression. Significant associations were found between PAWR protein expression and advanced clinical stage, nuclear grade and the presence of estrogen receptor. Furthermore, PAWR protein expression was directly associated with shorter disease and overall survival of the patients. Although further studies are required to better characterize the biochemical and biological function of *Pawr*, our results suggest that down-regulation of *Pawr* expression might be involved in the regulation of cell proliferation and survival by estrogens and growth factors, and provide evidence that increased PAWR protein expression play role in breast cancer progression and could serve as useful prognostic marker of the disease outcome.

Effect of 17b-estradiol (E2) and fulvestrant (ICI 182 780) ont the Pawr *mRNA expression in MCF-7 breast cancer cells. *, p<0.05 treatment groups that showed higher* Pawr *mRNA expression than the control group*



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HETEROLOGUS GENE EXPRESSION IN DIPTERAN CELLS: MOLECULAR BIOLOGY AND BIOPROCESS ENGINEERING

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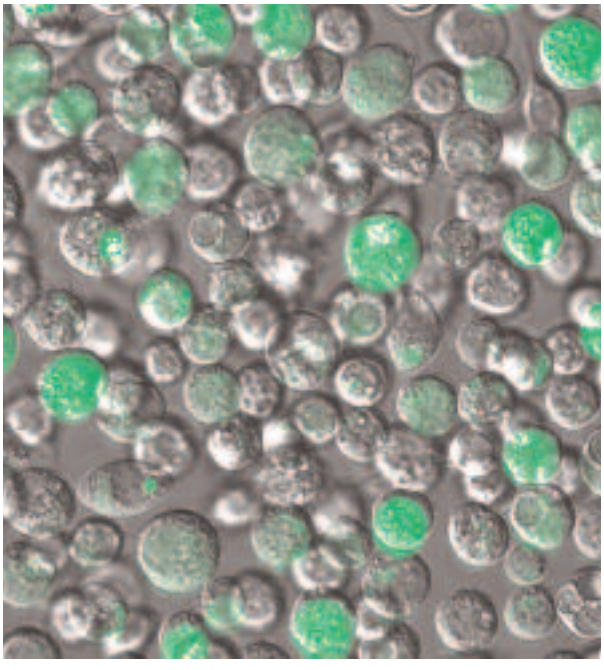

Drosophila melanogaster *cells expressing the rabies virus glycoprotein upon gene transfection*

The project assembles a multidisciplinary group constituted by biologists and chemical engineers from 5 different institutions in São Paulo State. The aims of the project are: the cloning of the rabies virus glycoprotein (RVGP) gene in expression vectors for the preparation of Schneider 2 (S2) *Drosophila melanogaster* cells stably transformed and expressing this gene, the optimization of a bioprocess for the production of the antigen in bioreactors, and the evaluation of its antigenic and immunogenic properties.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Plasmid vectors for S2 cells were constructed, characterized and used for cell transfection. S2 cells transfected with these vectors showed a high RVGP production (3 µg/10^7 cells) with enough quality to induce the antibody synthesis in immunized animals. We have formulated different S2 cell culture media and demonstrated that hemolymph and its fractions were capable of promoting cell growth and heterologous protein production. Optimized cell growth and metabolism have been obtained in bioreactors by controlling the cell sensitivity to hydrodynamic stress, the cell death and the RVGP production at the cell membrane. The RVGP synthesis parallels the cell growth and can be modulated. A higher hydrodynamic stress in bioreactors favors the recombinant protein production and the combination of this one with the medium formulation and alterations of the cell membrane are involved in a higher RVGP production. The S2 cell respiration, the dissolved oxygen concentration and the kLa of different systems of oxygen transfer were optimized for a higer RVGP production. A protocol for recombinant protein production by S2 cells was established and purification processes were developed. The RVGP produced was monitored by ELISA, flow cytometry, qPCR, confocal microscopy and western-blotting. *In vivo* assays showed a good immune response of mice to the RVGP. Quantitative and qualitative results of this project reinforce the good potential of S2 cells as a substrate for gene expression.

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PROTEIN FOLDING, STABILITY AND STRUCTURE

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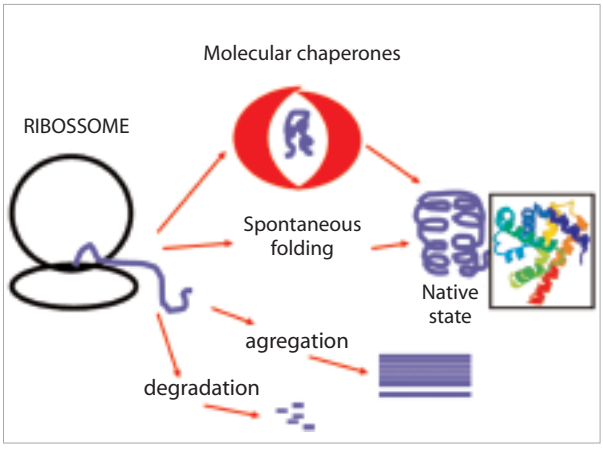

The fate of protein in the cell. Protein is produced by the ribossome as an extended polypetide that usually folds spontaneously to its native state. However, in certain conditions, proteins fold only partially generating aggregates which are unproductive because the function of most proteins is ordinarily related to its native conformation (some researchers think that misfolded proteins are the origin of, as much as, half of the human diseases!). Protein aggregation is worsened in stress conditions and molecular chaperones are the major factors that had enhanced expression during stress. Proteins that escape the initial action of chaperones and precipitate can be ressolubilized by other chaperones. Therefore, molecular chaperones constitute the central cellular defense against protein misfolding and aggregation that have major pathological consequences

The conversion of a polypeptide backbone into a native protein is a key element in the translation of the genetic information of an organism. As the organism ages, folding seems to deviate, which signals for several diseases (mainly neurodegenerative ones). Protein misfolding causes its deposition in the cell in the form of aggregates or amyloid fibrils, both of which have toxic effects. Molecules, that play an important role in cell protection, are molecular chaperones, which help protein folding and protein disaggregation. Therefore, chaperones seem to have a fundamental role in the organism by increasing the success of physiological functions and protecting cells from becoming ill. Our proposal has the main objective of understanding protein folding by: 1) studying the folding pathway and the stability of proteins, mainly globins; 2) characterizing the forces and the mechanisms of amyloid fibril formation; 3) structurally and functionally characterizing chaperones; and 4) studying the mechanisms by which chaperones help folding, stop aggregation, resolubilize aggregates, and interact with proteins involved in cell malignization.
Our goal is to understand protein folding inside the cell: such knowledge will generate important new ways of thinking, and may help lead to new therapies.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Human chaperones have been cloned and purified in their folded conformation, as measured by circular dichroism and fluorescence spectroscopy. The hydrodynamic properties of the proteins are under investigation by hydrodynamic techniques (analytical ultracentrifugation, gel filtration chromatography and dynamic light scattering). *Ab initio* calculations are underway and will give further insights on quaternary structure.

Xanthomonas secretion chaperone and target: two sets of secretion chaperones and their respective targets were selected by two-hybrid studies. Proteins were purified and their interaction measure by *in vitro* techniques.

Sugarcane chaperones: we initiated further characterization of chaperones that are part of the HSP70-HSP90 complex (involved in abiotic and biotic stress in plants – a pathogen causes biotic stress).

Results on protein folding showed that the information that is present on the amino acid sequence is also important to avoid aggregation. Permutation mutants may still exhibit native structure and function, but aggregate easily.

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MEMBRANE MICRODOMAINS ENRICHED IN (GLYCO) (SPHINGO) LIPDS AND STEROLS: ORGANIZATION, FUNCTION AND CELL SIGNALING

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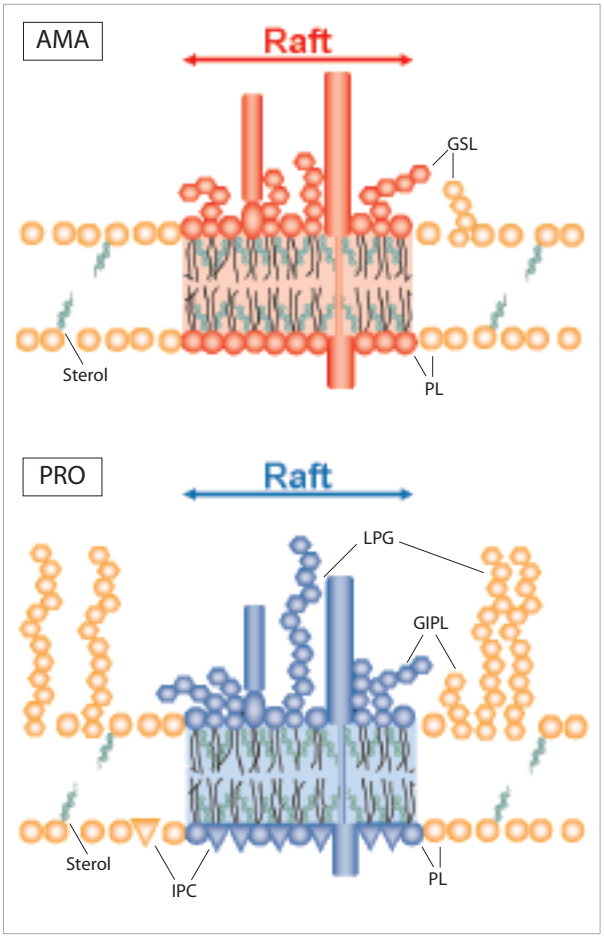

Hypothetical membrane rafts in L. (L.) amazonensis. *Lateral association between sphingolipids and sterols results in formation of rafts. Transmembrane and GPI-anchored proteins may serve as platform for cell signaling. GSL, glycosphingolipid; IPC, inositol phosphorylceramide; LPG, lipophosphoglycan; PL, phospholipid; GIPL, glycoinositolphospholipid*

In the past 10 years our laboratory have isolated and characterized various glycolipid antigens from *Leishmania*, *Trypanosoma cruzi* and pathogenic fungi. Glycolipid antigens are mainly located at the plasma membrane of these microorganisms, and recent results have shown the involvement of these molecules in processes of cell adhesion, recognition, and differentiation. The major objective of this project is to study, in details, the organization of lipids and glycolipids in microdomains at the plasma membrane, denominated membrane rafts, and their role in processes of adhesion, invasion, and survival of fungi and parasites in the mammalian host. Also, molecules from the host cells, presumably involved in the interaction with these pathogens, will be characterized, and the activated signal pathways will be analyzed in order to better understand the pathogen-host interaction. The specific objectives of this project are focused at determining and characterizing: i) components of the microdomains enriched in (glyco)(sphingo)lipids in trypanosomatids and pathogenic fungi; ii) interaction of fungi and trypanosomatids, as well as its purified glycoconjugates, with mammal cells; iii) structure and function of specific antigens of parasites and fungi, aiming to identify new target molecules for action of more specific drugs; iv) *in vitro* and *in vivo* effects of inhibitors involved in the biosynthesis of lipids, in the growth and infectivity of parasites and fungi; v) the functional and organizational dynamics of the microdomains in the pathogenesis of these microorganisms; vi) reorganization of membrane rafts and cytoskeleton during phase transition in dimorphic pathogenic fungi; vii) processes of cellular signaling in the interaction of parasite-cell host and fungi-host cell; and viii) (glyco)lipidomic analysis of fungi and parasites aiming to detect possible virulence markers.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Membrane rafts are cholesterol – and sphingolipid – enriched cell membrane domains, which are ubiquitous in mammals and play an essential role in different cellular functions, including host cell-pathogen interaction. By using several approaches, as localization in cell membrane of GM1, a membrane raft marker our group demonstrated that epithelial cell membrane rafts are essential for *Paracoccidioides brasiliensis* adhesion and activation of cell signaling molecules such as SRC-family kinases (SFKs).

Membrane rafts are also present in fungi. We observed that membrane domain fractions from *Histoplasma capsulatum* showed an enrichment of glycoinositol-phosphorylceramides, ergosterol, and proteins, such as PMA1P, a known yeast membrane raft marker. In addition, it was verified that disruption of fungal membrane rafts promoted an inhibition by 40% of *H. capsulatum* infection in alveolar macrophages, suggesting that integrity of protein and lipid organization of *H. capsulatum* membrane rafts is essential for yeast-cell interaction.

In a similar fashion, membrane rafts were demonstrated to be present in amastigotes (AMA) and promastigotes (PRO) of *Leishmania (Leishmania) amazonensis*, and promastigotes of *Leishmania (Viannia) braziliensis*. Regarding sphingolipids, promastigote membrane rafts present inositol phosphorylceramide (IPC) whereas membrane rafts of amastigotes of *L. (L.) amazonenis* present neutral glycosphingolipids. The disruption of these microdomains by incubating parasites with methyl-β-cyclodextrin inhibited significantly macrophage infectivity by *Leishmania*. Also the role of parasite sphingolipids in cell cycle, division and viability was analyzed by employing inhibitors of IPC synthase (Aureobasidin A, AbA) and serine palmitoyl synthase (myriocin). Aba completely inhibited growth of amastigote and promastigote forms of *L. (L.) amazonensis*, and myriocin promoted parasite cytokinesis inhibition. Taken together, these results indicate that (glyco)(sphingo)lipids are key molecules in the processes of invasion and infection of trypanosomatids and fungi.

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STUDY OF PHYSIOLOGICAL FUNCTION AND BIOTECHNOLOGICAL POTENTIAL OF PROTEASE INHIBITORS AND ANTI-HEMOSTATIC MOLECULES PRESENT IN HEMATOPHAGOUS ARTHROPODS

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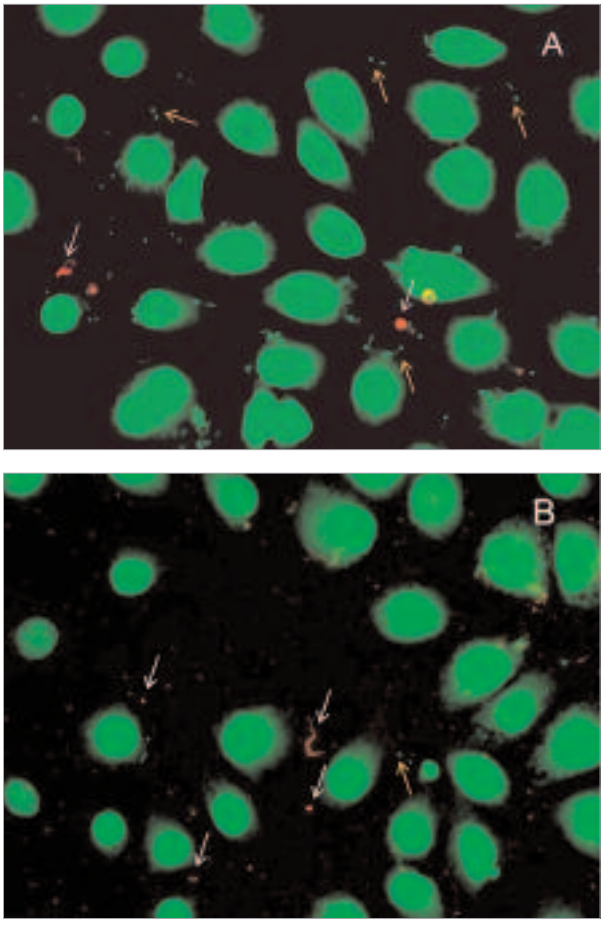

Inhibition of cell culture invasion by T. cruzi *strain strain Y using a* Triatoma infestans *protease inhibitor, Infestin 1R. The LLCMK2 cell line was infected using* T. cruzi *strain Y (1:10 parasites). A) Control experiment. B) Cells pre-incubated with 10 uM Infestin 1R before the infection with* T. cruzi *White arrows indicate the parasite outside the cell. Yellow arrows indicate the parasite inside the cell*

The biological molecule diversity present in hematophagous arthropods, in addition to their importance as disease vectors, has motivated our group to investigate new anti-hemostatic and protease inhibitors present in three different arthropod species, which are responsible for disease transmission to humans and animals in Brazil. They are: *Triatoma infestans* (kissing bug), *Boophilus microplus* (bovine tick), and *Aedes aegypti* (mosquito). In these three cases, the indiscriminate use of pesticide to control those vectors has caused contaminations to the environment and humans. Long term exposition of ectoparasites, to pesticides, has resulted in resistance problems. The World Health Organization has suggested that the main cause of failure in controlling these diseases is our ignorance about the biology of these animals. Therefore, a better understanding of several mechanisms that make these ectoparasites, successfully adapted for hematophagy, would help reveal new targets for developing control strategies, vaccines and also new drugs.

During the last eight years, our group has contributed to a better understanding of the biology of some hematophagous arthropods, among them the *T. infestans* bug, which controls its host's blood coagulation by thrombin and factor XIIa inhibitors that are interestingly coded by the same cDNA precursor molecule. Our studies using factor XIIa inhibitors allowed for a patent process application. We also showed several serine protease inhibitors isolated from *B. microplus* tick and *H. irritans* fly that could present useful physiological functions, such as controlling endogenous proteases and/or microorganisms. We thus aim at characterizing the function and structure of several molecules identified by our group, by using the following tools: RNA interference, real time PCR, proteomics, antifungal activity assays, microorganism protease inhibition, hemolymph coagulation, and activation of the phenoloxidase cascade. With these efforts, we intend not only to understand the role these molecules play in the hematophagous physiology, but also to use them for understanding arthropod protein-protein interaction, for vaccine development, and for biotechnological purposes in human and veterinary medicines and agriculture.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Recently, we have reached some goals including the characterization of three serine protease inhibitors isolated from *Boophilus microplus* tick eggs and larvae. The one named BmSI is a strong subtilisin A inhibitor, and is the first inhibitor which belongs to the TIL (Trypsin Inhibitory Like) inhibitor family to be described; and the others are similar to the Kunitz-BPTI family members (BmTI-6 and BmTI-A) as are practically all other trypsin inhibitors characterized from this tick up to the present time. At the beginning of this project, we finished the characterization of a cysteine protease inhibitor (Bmcystatin) isolated from the fat body of *B. microplus*, along with the characterization of a specific chymotrypsin inhibitor (BmCI) isolated from tick hemocytes. Its behavior after challenge with *Metarhizium anisopliae* fungi and its effects on eukaryotic cell lines suggested a possible role in the apoptosis process. The inhibitors BmTI-6 and BmTI-A are planned to be used in bovine immunization trials.

In addition, we characterized three important molecules from *Triatoma infestans* (kissing bug): (i) a new serine protease inhibitor, the TIPI1 (*Triatoma infestans* Pacifastin Inhibitor 1), the first Pacifastin inhibitor from a hematophagous insect to be described; (ii) a potent platelet aggregation inhibitor induced by collagen, named Tilipo 33, isolated from salivary glands (our results suggest that it targets a new collagen receptor on the platelet); (iii) a Kazal-type inhibitor from midgut, INF1R, which can interfere in the *T. cruzi* cell invasion by a still unknown mechanism.

Finally, the proteins Tilipo 33, Bmcystatin, and Cathepsin L have been used in crystallization experiments for further tridimensional structure determination.

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PEPTIDES AND PEPTIDASES: BIOLOGICAL ACTIVITIES IN INFECTIOUS DISEASES AND CANCER

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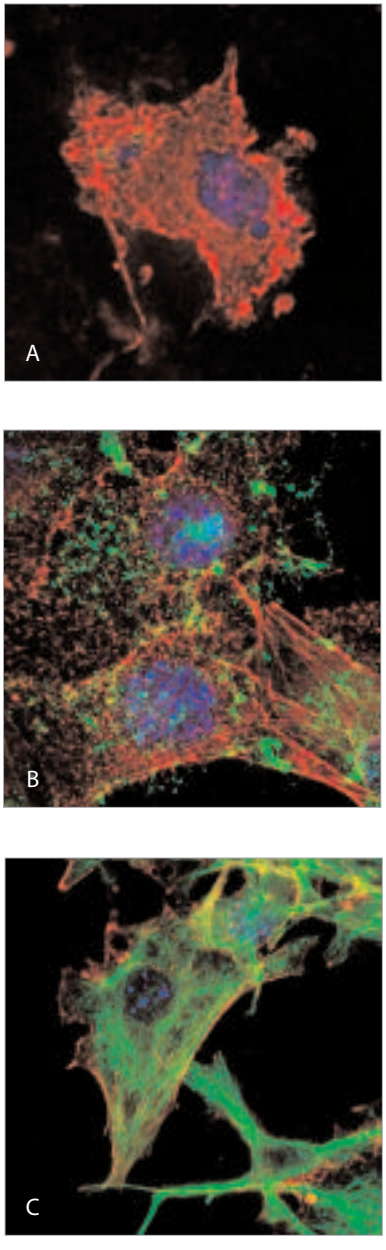


The main objective of the project is to focus on the synthesis and chemical characterization of peptides and derivatives. A series of sectional research lines focus on peptides involved in protective responses against eukaryotic pathogens and tumor cells.

Our research lines include: (i) the development of a peptide vaccine against paracoccidioidomycosis based on peptide P10 from the major diagnostic antigen gp43, (ii) the investigation of antitumor and antimicrobial peptide derived from hyper variable sequences of monoclonal Abs (CDRs), (iii) the study of anti-tumor activity of the permeabilizing peptide gomesin, (iv) the study of reagents able to neutralize the action of immunosuppressive cytokines, (v) the investigation of melanoma endooligopeptidases and cathepsins B, D, L and S as well as bacterial oligopeptidase A (OPDA) aiming at crystallization and (vi) specific aims on serine-, cysteine- and metallo-peptidases, kallikreins and ACE.

Gomesin accumulates on the cell membrane and permeabilizes B16F10-Nex2 tumor cells (examined by confocal microscopy). B16F10-Nex2 melanoma cells were cultivated on round glass coverslips, treated with 5 µM of gomesin for 10 min, fixed, and incubated with anti-gomesin antibody (B) or murine anti-tubulin antibody (C), both revealed with FITC-conjugated secondary antibodies, goat anti-rabbit and goat nti-mouse, respectively (green). Red, phalloidin-rhodamine and blue, DAPI staining. (A) Control, cells treated with Phalloidin-rhodamine, DAPI, anti-tubulin in the absence of gomesin. (modified from Neoplasia 10: 61-68)

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

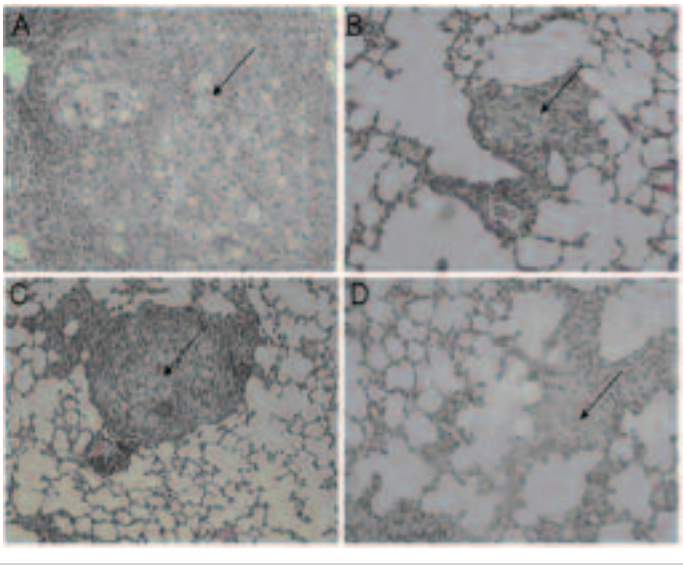
Solid phase peptide synthesis was used to obtain peptides that are purified and characterized by HPLC, mass spectrometry, peptide sequence and peptide analysis. Organic syntheses of peptidemimetics and non natural amino acids, as components of peptidase inhibitors, have also been in center of our work using classical organic synthesis methodologies.

Combinatorial peptide library approaches were also used as an alternative to the systematic assays of individual peptides for specificity determination of endoproteases. The combinatorial peptide library methods had involved their generation followed by screening to identify the optimal substrate sequences.

The lines of focused research were: Substrates and inhibitors for human proteases and pharmacological studies and substrates and inhibitors for proteases of parasites and virus related to human infectious diseases.

Our group had also described a novel and potential use for gomesin as a topical agent against unsuccessfully treated intradermal and epithelial skin cancers and we have shown that melanoma cells secrete endooligopeptidases which have an important role in tumor proliferation *in vitro* and *in vivo*.

Biochemotherapy with P10 and itraconazole against paracoccidioido-mycosis. Additive effect of P10 Immunization and Itraconazole, after 45 days, in anergic mice, infected intratracheally with 3x105 cells of Pb 18. A) Lung section from anergic mouse; B) Same, immunized with P10. C) Same, treated with itraconazole; D) Same, subjected to P10 immunization and drug treatment. HE staining was used, magnification, x10.



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INVOLVEMENT OF ENERGY METABOLISM, INTRACELLULAR Ca^{2+} HOMEOSTASIS AND OXIDATIVE STRESS IN CELL DEATH

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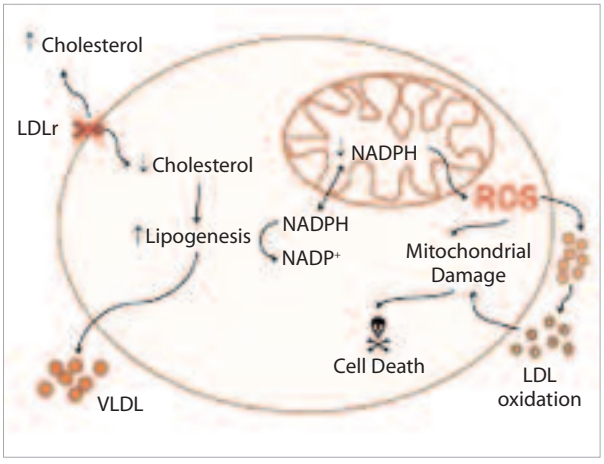


Mitochondrial RPS release in increased in LDL receptor knockout mice. (Vercesi et al., IUBMB Life 59: 263, 2007)

Many diseases are related to changes in energy metabolism, impairment of Ca^{2+}, Na^{+}, H^{+} or K^{+} homeostasis, increased reactive oxygen species (ROS) generation or the activation of mitochondrially-controlled cell death. Our Project seeks to enhance our comprehension of these diseases, studying mitochondrial oxidative metabolism in vitro and applying this knowledge towards the understanding of cellular and in vivo models of diseases known to be associated with energy metabolism defects, including dyslipidemias, atherosclerosis, stroke, methylmalonic acidemia and Parkinson's diseases. The main goals of our project are:

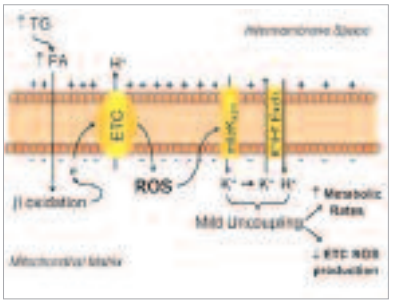
1. To verify the role of mitoK_{ATP} on the control of the mitochondrial redox state, oxidative metabolism, body mass and composition in genetically hypertriglyceridemic (hyperTG) mice;
2. To study the oxidative stress in cells and mitochondria of genetically hypercholesterolemic mice;
3. To understand the impact of the expression of the cholesteryl ester transfer protein (CETP) on the energetic metabolism and adiposity in CETP transgenic mice;
4. To study energy metabolism, oxidative stress and mitochondrial dysfunction in excitotoxicity and in dopaminergic neurodegeneration in Parkinson´s disease;
5. To characterize the mitochondrial dysfunction induced by methylmalonate;
6. Oxidative stress and mitochondrial dysfunction in lymphocyte death: influence of lymphocyte activation;
7. Oxidative stress in tumor cell death.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

1. The activation of the mitochondrial potassium channel (mitoK$_{ATP}$) previously observed in genetically hypertriglyceridemic mice seems to be a mechanism to protect these organelles against the oxidative stress normally associated with dyslipidemias.

2. The lower capacity of mitochondria from atherosclerosis-prone, hypercholesterolemic LDL receptor knockout mice to sustain a reduced state of matrix NADPH, the main source of antioxidant defense system against reactive oxygen, is the consequence of high rates of lipogenesis that consumes reducing equivalents from this coenzyme shifting it to the oxidized state.

3. In a recent work, we showed that two proteins involved in the plasma lipid transport, have opposite effects on the accumulation of body fat. The overexpression of apolipoprotein CIII causes hypertriglyceridemia and predispose to diet induced obesity. On the other hand, the overexpression of CETP (cholesteryl ester transfer protein) reverses the adipogenic effect of apolipoprotein CIII. These findings indicate a novel and unsuspected role for CETP in modulating body adiposity.



MitoKATP channels are activated in hypertriglyceridemia (Vercesi et al., IUBMB Life 59: 263, 2007)

4. We observed that *in vivo* overstimulation of N-methyl-D-aspastate (NMDA) receptors, by intracerebral infusion of sodium quinolinate in rat, causes an early impairment of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity. No impairment in mitochondrial Ca^{2+} accumulation at this stage of the degeneration was detected. Our results suggest that an early impairment of SERCA function may be involved in excitotoxicity.

5. In a recent publication, we showed that methylmalonate (MMA) is an important inhibitor of succinate transport by the dicarboxylate carrier. We concluded that MMA inhibits succinate-supported mitochondrial oxygen consumption by interfering with the uptake of this substrate. Although succinate generated outside the mitochondria is probably not a significant contributor to mitochondrial energy generation, MMA-induced inhibition of substrate transport by the mitochondrial dicarboxylate carrier may have important physiopathological implications in methylmalonic acidemia.

6-7. Activated spleen lymphocytes from Walker 256 tumor bearing rats are more susceptible than controls to necrotic cell death by a mechanism mediated by higher levels of cytosolic free calcium that enters mitochondria and stimulates the production of reactive oxygen. This may explain the increased fragility of the immunological system to chemotherapy-generated oxidative stress.

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IDENTIFICATION OF MOLECULAR MARKERS FOR DIAGNOSTICS AND PROGNOSTICS IN CANCER USING DNA MICROARRAYS

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During the development of the thematic project, *Identification of molecular markers for diagnostics and prognostics in cancer using DNA microarrays*, our group identified several long non-coding intronic transcripts, whose levels of expression were correlated to the degree of differentiation of prostate tumors. Results obtained more recently, from analysis of a set of 60 samples of prostate tumor from patients with or without relapse (metastasis), point to a signature of genic expression containing intronic RNAs correlated with the development of metastasis in that cancer. This result is extremely promising and opens the perspective for the development of new molecular tests for the prognosis of cancer of the prostate. Our group has also analyzed profiles of expression of intronic messages in other human neoplasms (kidney, breast, pancreas, leukemias). The results obtained suggest that intronic RNAs have a significant potential as diagnostic/prognostic markers in cancer.

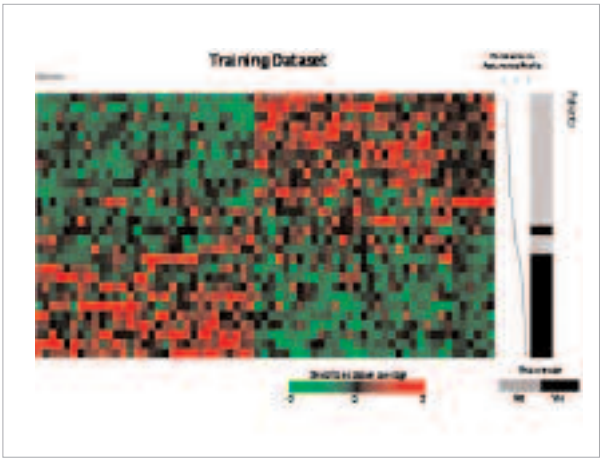

Figure 1 – The figure shows the expression profile of 65 genes (21 exonic protein-coding genes and 44 intronic noncoding RNAs) identified as most correlated to cancer recurrence (p-value <0.01 based on Signal-to-Noise Ratio analysis with permutations) in a set of 28 samples from patients with clinical follow-up longer than 5 years. The known clinical status of these patients (recurrence/non-recurrence) is shown on the right grey/black bar. The 65 genes are represented in the columns and each patient sample in one line. Patients are ordered according to the correlation value (r) of its profile with respect to the mean expression profile of patients with tumor recurrence. The expression level of each gene is represented as number of standard deviations above (red) or below (green) the mean expression of that gene across all samples

A recent bioinformatic analysis, by our group, revealed that around 75 per cent of human genes have evidence of intronic transcription. Based on this information, we designed a customized oligoarray, containing around 30 thousand probes, for intronic RNAs, which was used to analyze profiles of intronic expression in normal liver and kidney and tumors of the prostate. These experiments revealed the existence of signatures of tissue-specific intronic expression. Surprisingly, we observed that the most abundant of these intronic non-coding transcripts are generated in genic loci involved with the regulation of the transcription, in all three tissues. With the exonic sequences, we did not observe this phenomenon, which suggests a special role for intronic transcripts of the regulatory transcription genes, in the regulation of genic expression.

To explain the possible functional roles exercised by this new non-coding class of RNAs, we are testing *in vitro* the effect of modulators of genic expression on the intronic transcription. In this direction, we demonstrated recently, that the expression of a set of intronic transcripts may be modulated by the androgen hormone in a prostate lineage, and that these changes of expression affect the stability and/or pattern of splicing of the corresponding coding genes for protein.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Our project *Identification of molecular markers for diagnosis and prognosis in cancer using DNA microarrays* has identified a large number of long intronic noncoding RNA transcripts whose expression levels were correlated to the degree of differentiation of prostate tumors. Recent results have been obtained in the analysis of a set of 60 prostate tumor samples from patients with long follow-up (longer than 5 years) with or without recurrence (metastasis). The results point to a gene expression signature including noncoding intronic RNAs, that is correlated with recurrence of prostate cancer. This result is extremely promising and opens the way to developing new molecular tests for the diagnosis and prognosis of prostate cancer. Our group has been also analyzing the expression profiles of intronic RNA messages in other human neoplasias (kidney, breast, pancreas, leukemia). Our results suggest that intronic noncoding RNAs have a significant potential as markers of diagnosis and prognosis in all types of cancer.

A recent bioinformatics analysis of our group revealed that over 75% of all human genes have evidence of transcription of noncoding RNAs from their intronic regions. With this information, we have designed a custom oligoarray containing approximately 30 thousand probes for intronic noncoding RNAs that has been used to measure the intronic expression profile in normal liver and kidney human tissues as well as in prostate tumors. These experiments revealed a tissue-specific expression signature of intronic RNAs. Surprisingly, we observed that the most highly expressed intronic noncoding transcripts in each of the three tissues are mainly transcribed from gene *loci* involved with Regulation of Transcription (Nakaya et al., 2007). For exonic RNA this pattern was not observed, which suggests a special role of intronic noncoding RNAs in regulating gene expression.

In order to explore possible functional roles, exerted by this novel class of noncoding RNAs, we are testing *in vitro* cell cultures the effect of modulators of gene expression on the intronic transcription. We have recently demonstrated that the expression of a set of intronic noncoding RNAs can be modulated by androgen hormone in a prostate cell line, and that these changes of expression affects the stability and/or the splicing pattern of the corresponding protein coding genes.

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CHEMICAL AND BIOLOGICAL REACTIVITY AT INTERFACES

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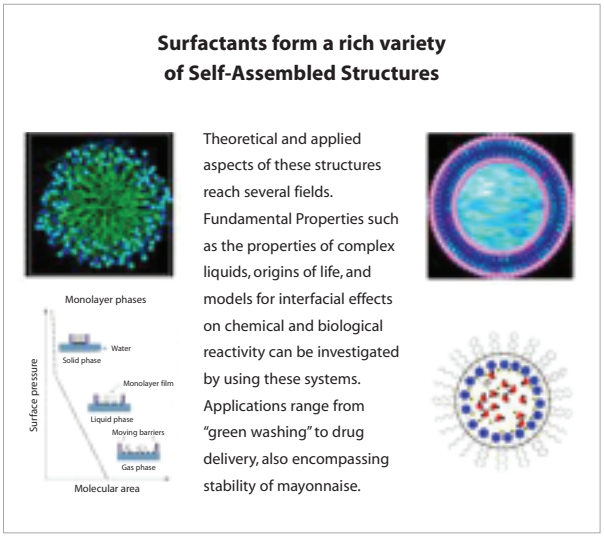

Figure 1

Essentially all chemical reactions in living systems occur at interfaces between protein and water or lipid and water. At a molecular level, interfacial properties change drastically with direction and distance, i.e., interfaces are highly anisotropic. Such features have intrigued and inspired scientists from many areas of research. Fundamental properties of organized systems and important practical applications have emerged from our increasing understanding of the exquisite properties of biological interfaces.

We have used association colloids, formed by self association of surfactants, to model interfacial propertie and intramolecular reactions to model enzyme catalysis.

Surfactants, of diverse chemical structures, are molecules with hydrophilic (water-loving) and hydrophobic (water-avoiding) parts. Depending on the hydrophilic/hydrophobic balance, the surfactants can self-aggregate to form aggregates exhibiting a rich architectural diversity (Figure 1). The delicate balance of forces that determine the self association of soap-like molecules into micelles, or lipid-like molecules into mono- or multilayers in water, is driven by opposing forces. The tendency of the hydrophobic part of the surfactant to either come out of solution (precipitate) or aggregate (hydrophobic effect) is the dominant factor for aggregation. The ensemble of steric and electrostatic repulsion forces resulting from the aggregation generally opposes the hydrophobic effect. This delicate balance of forces in surfactants' self-aggregation determines the diversity of architectures formed by these molecules in aqueous solutions. Understanding the connections between form and medium depends on precise knowledge of interfacial composition at the border between the aggregate and the surrounding medium and the specificity of the forces in that very limited space. The medium/form connection is a fundamental property equally important in biology as in nanosciences.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The relative acidity (pH) of the interface determines a variety of fundamental structural and functional properties. In probing the interface for acid-base dissociation, we demonstrated that in negatively charged interfaces modifications of local pH do not depend on changes in the equilibrium constant of weak acids (Figure 2). These observations can lead to applications such as the use of surfactants to protect from insecticides and nerve gases (Gonçalves et al, In Press, 2008). We have approached the investigation of the medium/form connections by observing the effect of interfaces on chemical reactions and using chemical trapping to directly determine interfacial composition. We recently demonstrated, by using appropriate mixtures of zwitterionic and positively charged surfactants, that the reaction rate, and thus the water concentration (or its hydrogen bonding ability), can be tuned at the interface by controlling the relative amount of surfactants in the mixture. It was evident from these results that strong ion-pairing can occur in water at the interface.

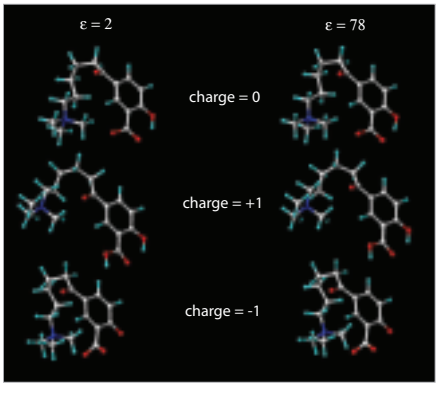

Figure 2. Preferential conformations of MHHB assuming different global charges (c) and dielectric constants (ε). (A) c = +1 and ε = 2, (B) c = +1 and ε = 78, (C) c = 0 and ε = 2, (D) c = 0 and ε = 78, (E) c=-1 and ε = 2, (F) c= -1 and ε = 78. pH at the micellar interface: Synthesis of pH probes derived from salicylic acid, acid-base dissociation in sodium dodecyl sulfate micelles and Poisson Boltzman simulation. J Colloid Interface Sci. ***297****:292-302.*

Modulation of ion pairing, at the interface, can produce shape changes, since the repulsive forces at the interface change under conditions in which the hydrophobic (attractive) component is not affected. In order to prove, the direct and simultaneous determination of water and counterion concentration at the interface of positively charged micelles. These measurements were possible using a chemical trapping method, developed by L. S. Romsted in the US, which was implemented and extended by our research group. An interesting model for shape change is the sphere to rod transition, observed when salt is added to solutions containing certain charged surfactants. The origin of life on earth is an intriguing problem that only very recently became a scientific question. In order to consider vesicles as models of inanimate objects in the evolutionary pathway, that led to life forms, some properties are required: catalysis, selectivity and size dependence. Using natural lipids and synthetic surfactants, we have demonstrated that vesicles can exhibit all these properties.

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PHOTODYNAMIC THERAPY (PDT): PHYSICAL, BIOCHEMICAL AND CLINICAL ASPECTS

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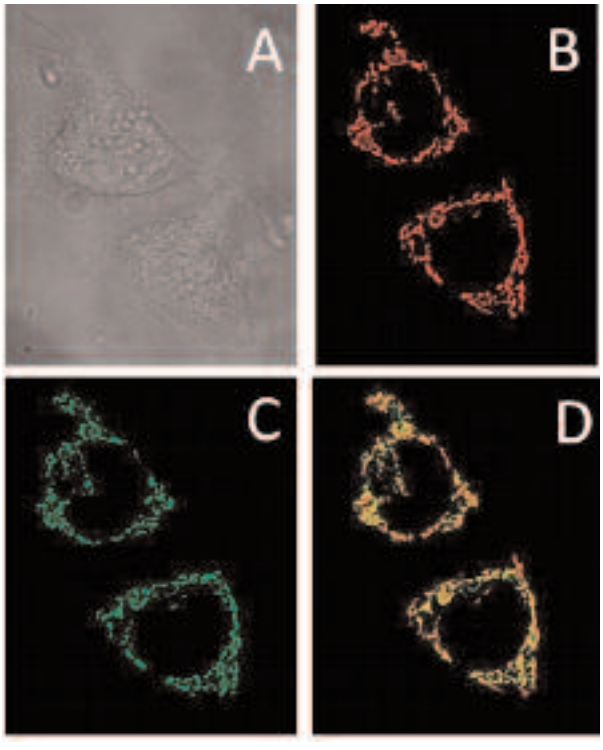

Micrographies of Hela cells: Transmitted light (A), fluorescence of PpNpNI (B), fluorescence of Rodamine 123 (C) and sobreposition of PpNpNI and Rodamine 123 (D).

The satisfactory outcome of a treatment by PDT depends on the interaction between light and living tissue, involving several processes that are usually dealt by professionals with expertise in different areas such as physics/photonics, chemistry/biochemistry and biology/medicine. These processes will be handled together on this project, by a team of researchers with multidisciplinary training. Our general aims are: i) to understand the physical, chemical, and biochemical mechanisms, ii) to synthesize, characterize and study activity of new potential drugs for PDT, iii) to propose low-cost clinical protocols using PDT for the treatment of cancer and infectious diseases; iv) to disseminate PDT to the various professionals in the health sciences, as well as to the general population. To achieve these goals, we propose the development of five sub-projects, which are: A) physical aspects that influence the efficiency in photodynamic PDT: penetration of light in living tissue and activation of photosensitizers; B) mechanisms in PDT: interaction of photo activated reactive species with biological systems; C) PDT *in vivo* and clinical trials; D) Synthesis of new molecules and photoactive nanomaterials; E) Development of tools to study photo damage in membranes. The mechanistic studies provide the necessary knowledge for the development of new drugs that will be synthesized and studied in the physical, chemical and medical aspects.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

Interfaces are known to affect the mechanisms of photochemical reactions and the photochemical reactions are known to affect the properties of the membranes. Our research efforts are directed to investigate some mechanistic aspects of these processes. We have shown how interfaces of biomimetic systems, mitochondria and nanoparticles can modulate the competition between type I and type II reactions, allowing photosensitizers to produce either singlet oxygen or radical species and examples were published of how this knowledge can be used to study photo oxidation reactions. By looking at the membranes, we have shown the relationship between chemical structure, membrane binding and photodynamic efficiency in cells, revealing the mechanism for the increased photodynamic efficiency of amphiphic photosensitizers. We have also shown how photo oxidation affects the cooperative interaction between cytochrome-c and membranes.

In order to amplify the knowledge relating chemical structure with photo-activity, we have made affords to synthesize new molecular photo sensitizer and nanoparticles modified with photosensitizers. By studying the photo activity of these species, in biomimetic systems and cell cultures, we aim to propose relationships between intensity and localization of photo oxidation damage, and the mechanism of cell death. This knowledge is important, not only to propose better protocols to PDT, but also to understand sun damage in skin. Although the final goal is not to search directly for more efficient photosensitizers, we end up discovering molecules that are more efficient compared to those commercially available, which could improve PDT protocols. Some results have been published and a patent application is being analyzed in the patent office of USP.

The understanding of photochemical processes in interfaces and membranes is often limited because of the lack of experimental tools that can be used to answer questions relevant to processes occurring few nanometers away from an interface. We have contributed to scientific knowledge in this area by developing tools to study photo oxidation processes in interfaces by using Surface Plasmon Resonance (SPR) and Giant Unilamellar Vesicles (GUVs). A membrane biochip was developed using SPR and a method to study physical damage in membrane was implemented using GUVs.

If the basic science that is learned in our experimental laboratories can be used to treat patients, the society will feel the direct benefit of our research. Therefore, we have made efforts to develop inexpensive PDT protocols and to offer then as a free service in hospitals and specialized clinics. Nowadays we are supporting clinical trials in a specialized dermatological clinic called CEDERM in UNIFESP (dermatoses and osteomyalites), in the Hospital Emílio Ribas (Leishmaniose), Medicine School of USP and Hospital Pérola Baiton (HPV, gynecologic cancer) and Medicine School of ABC (Kaposi´s Sarcoma).

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STRUCTURAL BIOPHYSICS OF NUCLEAR RECEPTORS AND RELATED PROTEINS

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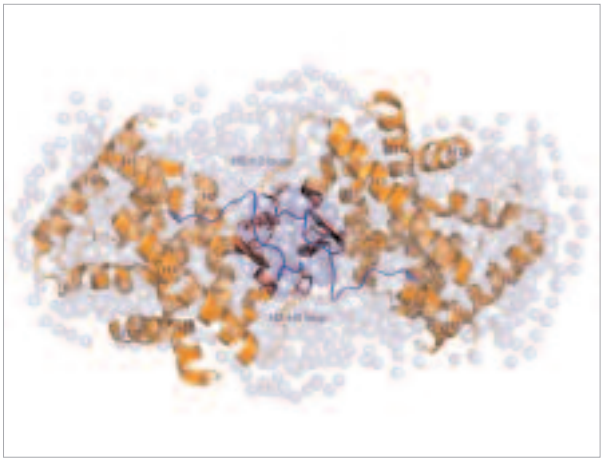


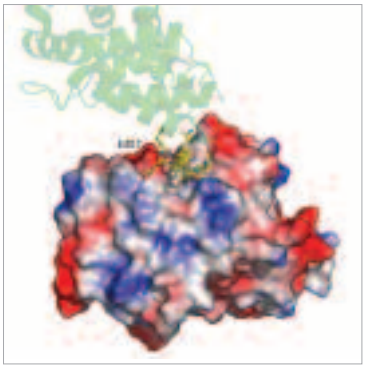
Low resolution shape of orphan nuclear receptor NGFI-B dimer superimposed with the crystal structures displaying a dimerization interface, which involves the H2-H3 loop

Nuclear receptors are among the most important intracellular regulating molecules that convey diverse internal and external signals into regulation of genetic programs. Genetic programming established or modified by these proteins affect virtually all the aspects of life of multicellular organisms. The intensive research on transcriptional regulation and selectivity of nuclear receptors nourished in attempts to decipher the complex network of molecular events involving such molecules. By unraveling the molecular rules, which define their control – both in space and time – over protein-protein and protein-DNA interactions, a myriad of possibilities could be opened up for developing more efficient drugs with superior therapeutical value.

Within the present project, we propose to study nuclear receptors by X-ray crystallography, small angle X-ray scattering (SAXS), biochemical and biophysical methods as well as by biocomputing simulations, in attempt to better understand what conformational changes of the receptor's 3D structures are induced by its binding to specific ligands (agonists and antagonists) and how this could influence the receptor's oligomeric state. In addition to those, we are also interested in characterizing its interactions with coregulating proteins (coactivators and corepressors), and also in modifying their stability. We aim at gaining insights into recognition of the nuclear receptors DNA response elements, by a combined approach of protein crystallography, SAXS and fluorescence anisotropy. Finally, we plan to study the role of nuclear receptor dynamics and flexibility in their function, seeking to determine the preferable dissociation pathways of the nuclear receptor ligands from their respective receptors by molecular dynamics simulations and experimental studies of the mobility and solvent accessibility changes by using hydrogen/deuterium exchange combined with mass-spectroscopy. It is important to stress that these aims have an immediate impact on the rational development of hormone agonists and antagonists, which are themselves poised for the scientific and technical development of Brazil in the area of nuclear receptors, and directly related to the interests of molecular biology, medicine and pharmaceutical industry.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

The main focus of research consists on structural, biochemical, biophysical and functional studies of nuclear receptors and related proteins. The structures of the ligand binding domain (LBD) of thyroid hormone receptor (TR) were determined and the role of the hinge domain was analyzed based on structural results and functional experiments. In addition, we conducted molecular dynamics computational studies of the escape pathways of ligands from TR LBD. We demonstrated that, contrarily to what is being currently accepted in the area, there are at least three dissociation pathways of ligands from TR, and that the "canonic" escape path, via helix 12, is not the preferred one.



Hinge-domain (H0) of thyroid hormone receptor TR is capable of docking of its co-activator binding groove

Furthermore, by using a combination of biophysical and biochemical techniques we have established for the first time that TR can form tetramers in solution. We reconstructed low-resolution models of TR dimers and tetramers in solution by using small-angle X-ray scattering (SAXS) and then went on to discuss physiological implications of the multimeric forms of thyroid receptor. We determined the X-ray structure of another nuclear receptor, PPARγ LBD complexed with a synthetic ligand derived from canabinoid acid, which revealed that the ligand binds PPARγ at two binding sites, in the interior and on the surface of LBD. Biophysical studies of NGFI-B by SAXS and hydrogen/deuterium exchange, followed by mass-spectrometry, demonstrated that this orphan nuclear receptor forms dimers through non-canonical dimer interface which is similar, but not identical, to the glucocorticoid receptor (GR) dimer interface. This might explain competition between these two receptors in cells.

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MOLECULAR BASIS OF DIFFERENTIATION OF STEM AND NEURAL PROGENITOR CELLS

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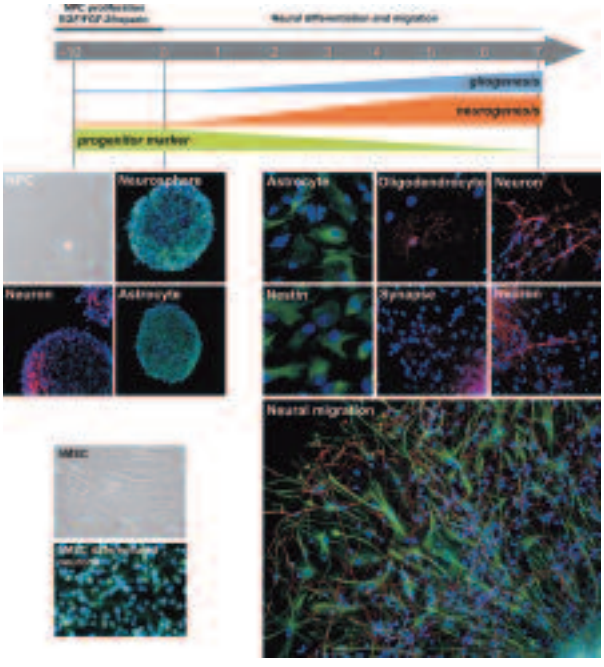


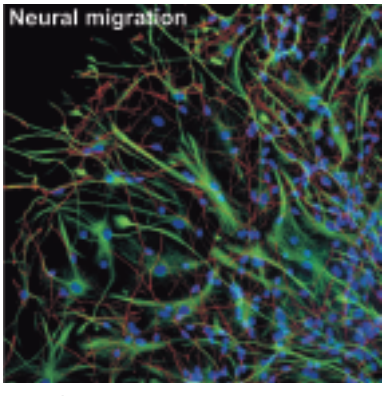
The immense phenotypic variety of cells in the central nervous system (CNS) is due to the differential development of stem and progenitor cells which give origin to neurons, astrocytes and oligodendrocytes

The nervous system is formed by a network formed by trillions of neurons with different phenotypes and other supporting cell types. The process of neurogenesis is directed by the activation of innumerous receptors on cell surfaces of differentiating cells. We have shown in previous studie the participation of kinin-B2, purinergic and cholinergic receptors in neuronal differentiation using P19 embryonic carcinoma cells as an *in vitro* model.

Cholinergic receptor expression was altered when cells were differentiated in the presence of inhibitors of purinergic or kinin-B2 receptors. Based on these results, we are proposing to study the participation of these receptors in the neural differentiation of mouse embryonic and mesenchymal stem cells, as well as, in the process of maturation of rat progenitor cells (neurospheres). DNA aptamers will be identified using the SELEX technique from a combinatorial DNA library, which is capable to specifically recognize stem cells, in order to purify these cells from contaminating ones. Gene expression and receptor activities of kinin-B2, purinergic and nicotinic receptor activity, during differentiation of embryonic and mesenchymal stem cells and neurospheres, will be determined. The fates of neuronal differentiation will be evaluated in the presence of a specific antagonist of kinin-B2 receptors and subtype-specific inhibitors of muscarinic and nicotinic acetylcholine receptors. RNA aptamers and RNA interference will be used for subtype-specific inactivation of purinergic P2Y1, P2X2,4,7 receptor activity or gene expression, respectively. This study will contribute to the functional analysis of stem cells differentiated *in vitro*, and furthermore, to verify the participation of kinin-B2 and purinergic receptors in directing neuronal differentiation. Furthermore, the capability of stem and progenitor cells to give origin to regenerative processes, in the peripheral nervous system, will be evaluated.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

By using P19 embryonic carcinoma cells as an *in vitro* model, we have demonstrated the participation of kinin-B2, purinergic and cholinergic receptors in neuronal differentiation. Cholinergic receptor expression was altered when cells were differentiated in the presence of inhibitors of purinergic or kinin-B2 receptors. Nicotinic acetylcholine receptors were active during all stages of neuronal differentiation, including in embryonic cells, whereas muscarinic receptor function was only identified beginning from the neural progenitor stage. Muscarine induced proliferation in progenitor cells, by activation of Gαq/11-coupled M1, M3 and M5 receptors, whereas Gαi/o-protein M2 receptors contributed to acceleration of differentiation in the presence of muscarine. Purinergic receptor expression was modulated during differentiation, and P2Y1 and P2Y2 receptors participated in proliferation and differentiation induction of embryonic and neural-progenitor P19 cells as judged from pharmacological analysis. Kinin-B2 receptor expression and activity, as well as bradykinin secretion into the culture medium, increased during the course of neuronal differentiation. These studies led to conclude that numerous ionotropic and metabotropic receptors interact in a network and are activated at specific checkpoints of differentiation, thereby contributing to a pattern of calcium transients involving influx of extracellular calcium as well as calcium, mobilization from intracellular pools. Based on the results obtained with P19 cells, the objective of the on-going thematic project is to study participation of these receptors in the neural differentiation of mouse embryonic and mesenchymal stem cells, as well as, in the process of maturation of rat progenitor, cells (neurospheres). We could already identify the presence of components of the kallikrein-kinin system as well as bradykinin secretion and active kinin-B2 receptors during neurosphere differentiation. Treatment of differentiating neurospheres, with the kinin-B2 receptor inhibitor HOE-140, resulted in a loss of purinergic receptor activity. Ongoing studies will reveal the functions of cholinergic, purinergic and kinin receptors in neural differentiation of embryonic and mesenchymal stem cells, as well as, of neural progenitor cells, thereby providing insights in how to direct differentiation to a specific neural phenotype.



Neural migration

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IONIC CURRENTS AND RECEPTORS IN THE PHYSIOLOGY OF EXCITABLE AND NON-EXCITABLE CELLS

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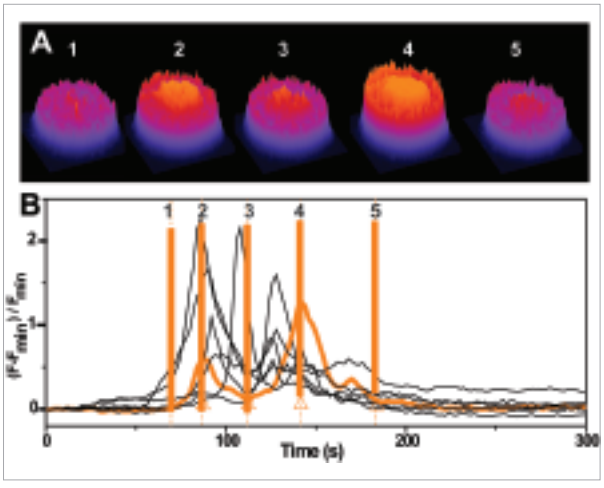
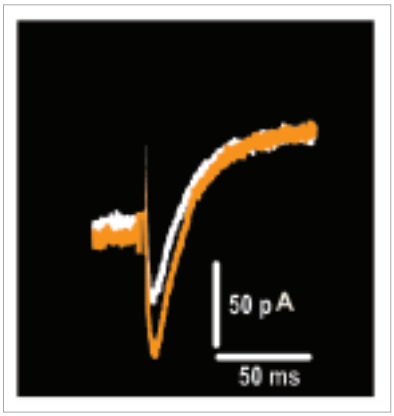


Calcium signals in Leydig cells. A Fluorescence signals in a Leydig cell in control condition (1) and after treatment with 1 Ìg/ml of LH (2, 3, 4 and 5). B Time course of the fluorescence change for several cells (each trace correspond to a particular cell). Vertical bars and orange trace correspond to the specific times (1, 2, 3, 4, 5) where fluorescence was measured in the cell shown in A. Cells were loaded with the calcium sensitive dye fluo-3 (Molecular Probes) and the experiment was made on a Leica SP5 confocal microscope. Images processed using ImageJ software (http://rsb.info.nih.gov/ij/)

This project is concerned with ion transport in Leydig cells and in cells of neural tissue (supra optic nucleus) aiming at an understanding of the role played by specific receptors and ionic currents in the physiological processes carried out by these cells. Although both cells are involved in secretor processes, testosterone in one case and ADH in other, we intend to use the patch clamp technique in order to get information about the major ions participating in their physiology, coupled to current molecular biology techniques to identify specific receptors and calcium ion fluorescence to evaluate changes in the intracellular concentration of this ion. The expected results should contribute to the understanding of the secretor processes, important for the determination of the male sexual characteristics in one case and for the maintenance of the osmolality of the extracellular fluid in the other: both crucial phenomena for the maintenance of life. Under a theoretical perspective, we intend to use mathematical tools (Hiden Markov Chains) to analyze single channel ionic currents through calcium activated potassium channels, known to be expressed in Leydig cells. The expected results should contribute to the understanding of the physical mechanisms controlling the transitions between the closed and opened conformation of the protein forming the channel.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

We are devoted in understanding the mechanisms by which ion channels and receptors respond to mediators and are controlled by and participate in the cell physiological processes. We use two distinct cells in our experiments: 1) In magno cellular neurons of the supra-optic nucleus, electrophysiological results indicate that Nitric Oxide have a direct effect by modulating their firing rate and therefore the secretion of Vasopressin and/or Oxytocin and 2) In mice Leydig cells, we have shown, with the patch clamp technique, that ATP receptors, probably heteromers of the P2X2/4/6, subtype are present and involved in mediating calcium entry when stimulated. Volume Regulated Anion Channels (VRAC) is also present and may represent a pathway for ATP efflux, suggesting their participation in an autocrine control of testosterone secretion. Despite the well known fact that calcium ions are important in the response of the cells to Lutheinizing Hormone (LH), the intracellular pathways involved are still controversial. In this project we also investigate the presence and participation of T-type calcium channels in this response and correlate them with the overall intracellular calcium transients measured with fluorescent dyes and confocal microscopy. The ion current carried by the T-type calcium channels are stimulated by cAMP and lead to the activation of intracellular ryanodine and IP3 receptors with the consequent release of calcium ions from these intracellular stores. Since LH also leads to membrane depolarization (measured with fluorescent dye) and opening of the calcium channels, we are working with the hypothesis that this calcium current may function as the trigger for the intracellular events that follows.



T-type calcium currents in Leydig cells. Current recordings in response to a depolarizing voltage pulse from -80 to -20 mV under control condition (with trace) and after treatment of the cell with 1 Ìg/ml of LH. Note the increase in the peak current induced by LH. Data was obtained with the whole cell configuration of the patch clamp technique

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