UNIVERSITA' degli STUDI di NAPOLI
FEDERICO II

Facoltà di Farmacia
Department of Experimental Pharmacology

PhD THESIS in
'Scienza del Farmaco'
XIX CYCLE

Effects of inflamed *status* on functional and molecular vascular reactivity

Supervisor
Prof. Sorrentino Raffaella

Coordinator:
Prof. Enrico Abignente

Doct. Sorrentino Rosalinda

2003-2006

Appendix

I. Introduction

Cardiovascular diseases are the major causes of mortality and morbidity in the western society. Diabetes and sepsis are the main pathologies I have been focused in during my PhD training. They are two different cardiovascular diseases and in particular could be classified respectively into chronic and acute vascular pathologies.

Diabetes is a metabolic syndrome, characterized by persistent hyperglycaemia, increase of blood glucose which is not metabolized by the cell as a decrease or inefficiency of the insulin. Insulin is a pancreatic hormone responsible of the transfer and therefore catabolism of glucose from the blood into the cell. The diabetes can be classified in type I or mellitus, insulin-dependent, or type II, non-insulin-dependent. In the first case, pancreatic β-cells are not able to produce insulin as an autoimmune defect can occur to destroy the pancreas; in the second case, the insulin is normally produced but it is not recognised by the target cells, so that this pathology is considered as an insulin resistance. One of the major concerns related to the diabetes is the development of micro- and macrovascular complications, which contribute greatly to the morbidity and mortality associated to the disease. Microvascular complications are related to diabetic retinopathy, nephropathy and neuropathy, whilst macrovascular complications are related to hypertension and atherosclerosis, which are the most life threatening aspects of this cardiovascular disease.

Sepsis instead, is a serious medical condition resulting from the immune response to a severe infection. The immunological response that induces to sepsis is a systemic inflammatory response causing a widespread activation of inflammation and coagulation pathways. This may progress to dysfunction of the circulatory system

and, even under optimal treatment, may result in multiple organ dysfunction syndrome and eventually death.

Sepsis and diabetes could be described as acute and chronic cardiovascular diseases. We could consider sepsis as an acute cardiovascular disease as it has a rapid onset on the cardiovascular system, whereas diabetes could be ascribed to a chronic pathology as its cardiovascular dysfunction are long-lasting and are secondary to the metabolic syndrome. Beyond the effective causes, both pathologies have a common underlying matrix which is the reflection of the main cause onto the cardiovascular system: diabetic patients are subjected to high blood pressure, due to a high sensitivity to vasoconstrictors; whereas septic patients are subjected to low blood pressure, due to the low reactivity to vasoconstrictors and high susceptibility to endogenous vasodilators.

Furthermore, they could be classified as inflamed *status*. Sepsis is directly caused by an infection that can immediately induce an inflammatory process consequently recruiting inflammatory cells, whilst the inflammatory pathway during diabetes is secondarily activated by the 'shear stress' provoked by the hypertension, which induces endothelial and vascular smooth muscle impairment. The progressive inflammatory damage can then macroscopically reflect on the cardiovascular system. In the case of sepsis, the immediate vasodilatation and low blood organ perfusion is provoked by the high out-put of cytokines, chemokines and inducible enzymes, like iNOS or COX-2, after the infection challenge; whereas, in the case of diabetes, the indirect oxidative stress, caused by the high blood pressure and the consequent 'shear stress', can activate in the same way the overproduction of cytokines and chemokines, altering the already injured cellular physiological balance.

In the first part of my PhD I have been focused on the effect of the inflammatory *status* on several cell types, in particular studying the effect of bacteria challenge in terms of cytokines, chemokines and nitric oxide production, trying to explain the typical redundant inflammatory pattern during sepsis. Secondly, I analyzed the effect of LPS administration on the cardiovascular system reporting either *in viv*o or *ex vivo* evidence for the low vascular reactivity to vasoactive agents. I focused the attention on a possible coadjuvant for sepsis therapy combating the well-known vasodilatation, low blood pressure and endothelium impairment. In the end, I evaluated the vascular effect of a new gastransmitter, hydrogen sulphide, on vessels like aorta and mesenteric bed, representing capacitance and resistance vessels, respectively, in a rat model of type I diabetes.

Chapter 1

Gram positive bacteria versus Gram negative bacteria on human lung epithelial cells: effect on the chemokine-8 (CXCL-8)

1.1 Introduction: sepsis

In 1879-1880, Louis Pasteur showed for the first time that the invasion of a pathogen in a human organism can induce a severe systemic inflammation.

Sepsis is clinically defined as the presence of an infection, accompanied by a systemic inflammatory response.

The symptoms of the systemic response are:

1) temperature greater than 38°C or less than 36°C;

2) pulse rate greater than 90 beats/min;

3) respiratory rate greater than 20 breaths/min;

4) white blood cell count greater than 12000/mm^3 or less than 4000/mm^3 (1).

There are several definitions for describing the different severe stages of the sepsis; endotoxemia is the result from both local or systemic Gram-negative bacteria infections and from translocation of the whole bacteria or endotoxin from the gut into the circulation (2); it can convert to severe or septic shock and induce hypotension, multiple organ dysfunction, like acute lung injury, coagulation abnormalities, thrombocytopenia, altered mental status, renal, liver or cardiac failure. Normally, the immune and neuroendocrine systems tightly control the local inflammatory process to eradicate invading pathogens. When this local control mechanism fails, systemic inflammation occurs, converting the infection to sepsis, severe sepsis, or septic shock.

Sepsis can be induced by Gram negative, Gram positive bacteria or by polymicrobial infections. The most common bacteria are *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pneumoniae* (Table 1).

	Estimated Frequency
Gram positive bacteria	30-50%
Meticillin-susceptible *S.aureus*	14-24%
Meticillin-resistant *S.aureus*	5-11%
Streptococcus pneumoniae	9-12%
Enterococcus	3-13%
Anaerobes	1-2%
Gram negative bacteria	
E.coli	9-27%
Pseudomonas aeruginosa	8-15%
Klebsiella pneumonite	2-7%
Haemophilus influenzae	3-7%
Fungus	
Candida albicans	1-3%
Yeast	1%
Parasites	1-3%
Viruses	2-4%

Table 1: Main pathogens responsible of septic shock induction.

1.1.1 Sepsis: effect on the lung

The most common infectious sources of severe sepsis are the lungs, abdomen, and urinary tract. In this study we have been focused on the effect of Gram positive and Gram negative bacteria effect on the lung and in particular on human lung epithelial cells. It is well established the effect of Gram negative bacteria, like E.*coli*, on the induction of sepsis, but there is an increasing evidence of Gram positive bacteria implication in the physiological airway function (1).

Clearance of bacterial pathogens from the lung is largely dependent upon effective innate immune responses. Macrophages are important innate immune cells and play a critical role in host defence against bacterial pathogens in the lung. Alveolar macrophages are capable of ingesting and eradicating bacteria that reach the terminal airspaces. When the number of bacteria overwhelms the macrophage's bactericidal capabilities, the ability to mount an effective antimicrobial response requires the cytokine-mediated recruitment of neutrophils. Neutrophils are important for early control of acute bacterial infections and thus are considered pivotal to protective innate immunity. Neutrophils are a type of leucocytes that are attracted to the site of infection to phagocyte the pathogen. They adhere to bacteria by an Fc receptor that recognizes the constant chain of the immunoglobulins that have opsonized or covered the bacteria. Consequently, they prevent spread of micro-organisms by phagocytosis and release of toxic agents such as pro-inflammatory cytokines, proteases, and reactive oxygen species.

1.1.2 Innate immune system

Innate immunity initiates immediate defence mechanisms on the basis of non-clonal recognition of microbial components. Identification of microbes by these

mechanisms involves host receptors, termed pattern recognition receptors or PRRs that recognize conserved molecular motifs on a wide range of different microbes. These motifs have been termed 'pathogen associated molecular patterns' or PAMPs and include such structural molecules as flagellin, peptidoglycan, lipopolysaccharide (LPS) and double-stranded RNA (3). Toll-like receptors (TLRs) and nucleotide binding oligomerization domain (NOD) proteins are two classes of PRRs involved in innate immune detection. TLRs are a family of membrane bound receptors, whereas NOD molecules reside within the cytoplasm and detect microbial motifs that gain entry into the host cell.

1.1.2.1 Toll like receptors

In 1991, a homologue receptor of proinflammatory interleukin-1 (IL-1) was found. This was a receptor in *Drosophila melanogaster* termed *Toll*. The cytosolic portion of Toll was shown to be highly similar to the cytosolic portion of IL-1 receptor, although extracellularly they were quite different. This domain was subsequently named the Toll/IL-1 receptor (TIR) domain.

The discovery of TLRs has opened up a whole new range of therapeutic possibilities, largely for infectious diseases and sepsis, but also for inflammatory diseases and vaccine development (3).

TLRs are expressed at the surface of several immune cells and other cell types in areas of the body that are normally sterile. Thirteen members of the TLRs family have been identified in mammals and only 9 are functional (4). TLR1, TLR2, TLR4, TLR5 and TLR6 are all localized to the plasma membrane whereas TLR3, TLR7, TLR8 and TLR9 are preferentially expressed in intracellular compartments such as endosomes (4). TLR3, TLR7, TLR8 and TLR9 all recognize nucleic acid

structures from bacteria and virus, whereas TLR1, TLR2, TLR4, TLR5 and TLR6 generally recognize cell wall components.

Four adapters are known to mediate TLRs signalling and share significant amino-acid sequence similarity within their TIR domains (4), including myeloid differentiation factor 88 (MyD88), TIR-domain containing adapter protein (TIRAP), also called MyD88 adapter-like (Mal), TIR domain-containing adapter inducing interferon beta (TRIF), also called TIR-domain containing adapter molecule 1 (TICAM-1) and TIR containing protein (TIRP), also referred to as TRIF-related adapter molecule (TRAM) or TICAM-2.

Several evidences in literature indicate that TLRs pathways consist of a MyD88-dependent pathway that is in common to all TLRs, and a MyD88-independent pathway that is peculiar to the TLR3 and TLR4 signalling pathways (5).

After stimulation, MyD88 recruits several isoforms of IL-1R-associated kinase (IRAK) like IRAK4, particularly important for the responses to IL-1 and ligands that stimulate various TLRs. Activated IRAK then associates with tumour-necrosis factor (TNF) receptor-associated factor-6 (TRAF-6) leading to the activation of c-jun N-terminal protein kinase (JNK) and nuclear factor (NF)-kappaB-dependent pathways, which in turn regulates the expression of several genes involved in the inflammatory response, such as cytokine secretion as IL-6 and TNF-α.

The 'MyD88-independent' pathway exists downstream of LPS/TLR4 and double stranded RNA/TLR3 (4). Although MyD88 knockout mice are impaired in LPS-induced NFκB activation and the production of several cytokines, the production of interferon-β and likely other genes that are regulated by IFN regulatory factor-3 (IRF-3), a transcription factor necessary for the expression of IFN- β and RANTES

genes, are perfectly normal in these mice (6), confirming a different and MyD88-independent signalling pathway.

TIRAP or Mal was the second adapter protein to be identified in TLRs signalling pathway. It is specific for TLR2 and TLR4 and acts in conjunction with MyD88. TIRAP/Mal appears to homodimerize and to associate constitutively with TLR4 but not with TLR9, which uses the only MyD88 for its downstream signalling transduction (7). TLR2 can form heterodimers with TLR1 or TLR6; thus, TIRAP/Mal might bind to TLR1 and TLR6 to facilitate the association.

The third adapter protein is TRIF or TICAM-1 and it is responsible for the induction of MyD88-independent pathway (5). TRIF-deficient mice are defective in TLR3 and TLR4 production of IFN-β (5). Moreover, mice deficient in both MyD88 and TRIF show loss of all LPS-induced responses indicating that only MyD88- and TRIF-dependent signalling originate from TLR4 (5). Furthermore, TLR3 is unique in not requiring MyD88 to mediate downstream signalling pathways (5).

Finally, a fourth adapter protein TRAM or TICAM-2 appears to play a role, along with TRIF, as a co-adapter in the MyD88-independent component of the TLR4 signalling pathway (5). It is TLR4 specific.

In response to LPS stimulation, the two TLR4 molecules are brought into juxtaposition, permitting homodimerization of TICAM-2 through their loop cysteine residues. MyD88 interacts directly with TIRAP/Mal (4) and promotes the phosphorilation of IRAK4, a downstream serine/threonine kinase, which is able to connect with IRAK1. This complex engages with TNF-receptor-associated factor-6 (TRAF6) and thereby the activation of IKK through a TAK1-TAB1-TAB2 kinase complex. Once activated, TAK1 phosphorilates the activation loop of IKK β, thereby activating the IKK complex and the pleiotropic transcription factor, NF-κB.

TAK also phosphorilates MKK6 and 7, which in turn activate the p38 and the JNK kinase pathways, respectively (8). NF-κB activation results in the production of pro-inflammatory cytokines, dendritic cell maturation and the up-regulation of co-stimulatory molecules (9).

TLR2 recognizes a vast array of microbial components including lipoproteins from various pathogens, PGN from Gram positive bacteria, glycophosphatidilinositol (GPI) anchors from malaria and forms from LPS that are distinct from those recognized by TLR4 (10). The ability to recognize such a wide range of compounds has been attributed to the ability of TLR2 to heterodimerize with TLR1 and 6. The association with TLR1 permits the recognition of triacyl lipopeptides whereas TLR2/6 recognizes diacyl lipopeptides. TLR2 utilizes TIRAP/Mal and MyD88 to transmit signals to NF- κB. The phosphorilation of TLR2 on specific tyrosine residues facilitates the recruitment of phosphatidil inositol-3-kinase (PI3K) which activates, in turn, Rac-1, a small G-protein, that binds NF- κB into the nucleus (11). (Fig 1)

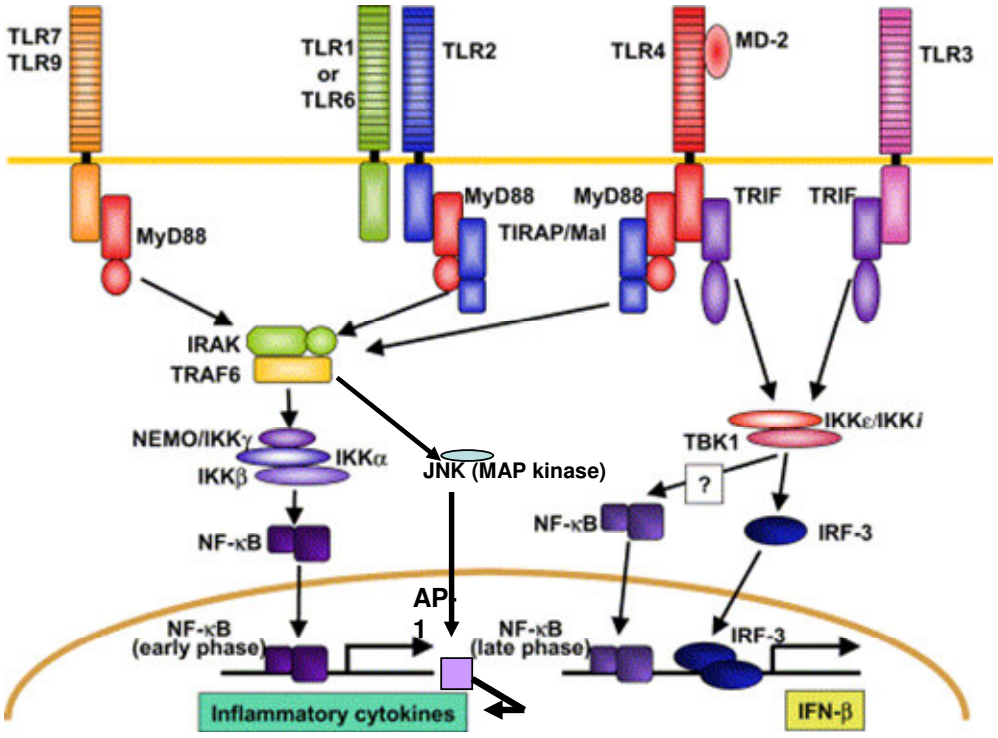

Fig. 1 TLRs signalling pathway

1.1.2.2 Nods proteins

Mammals have two closely related NOD family members — NOD1 and NOD2 — both of which contain one or two caspase-activating and recruiting domains (CARD), respectively, a central NACHT domain and C-terminal leucine-rich repeats (LRRs) (12). Nod1 and Nod2 proteins are involved in the intracellular sensing system of bacteria.

Nod1 also called CARD4 (caspase-activating and recruiting domain-4), is ubiquitously expressed in adult tissues and mainly 'senses' products from Gram negative bacteria, selectively recognize γ-D-glutamyl-*meso*-diaminopimelic acid (ie-DAP), another cell-wall derivative from Gram-negative bacteria (13).

Nod2 is characterized by the CARD15 domain and recognizes the muramyl dipeptide (MDP), a minimal cell wall component from Gram-positive and -negative bacteria (14). It would seem similarly advantageous to also have a mechanism to identify intracellular pathogens that sneak by the early-warning systems and reside, proliferate and transit through cells.

The binding of the PGN-derived products to Nods proteins induces a conformational change of these proteins which leads to the interaction CARD-CARD, involving the activation of the kinase RICK. RICK is a CARD-containing serine/threonine kinase that physically associates with CARD of Nods and induces the phosphorilation of IKKγ, inducing NF-kB translocation to the nucleus (Fig 2).

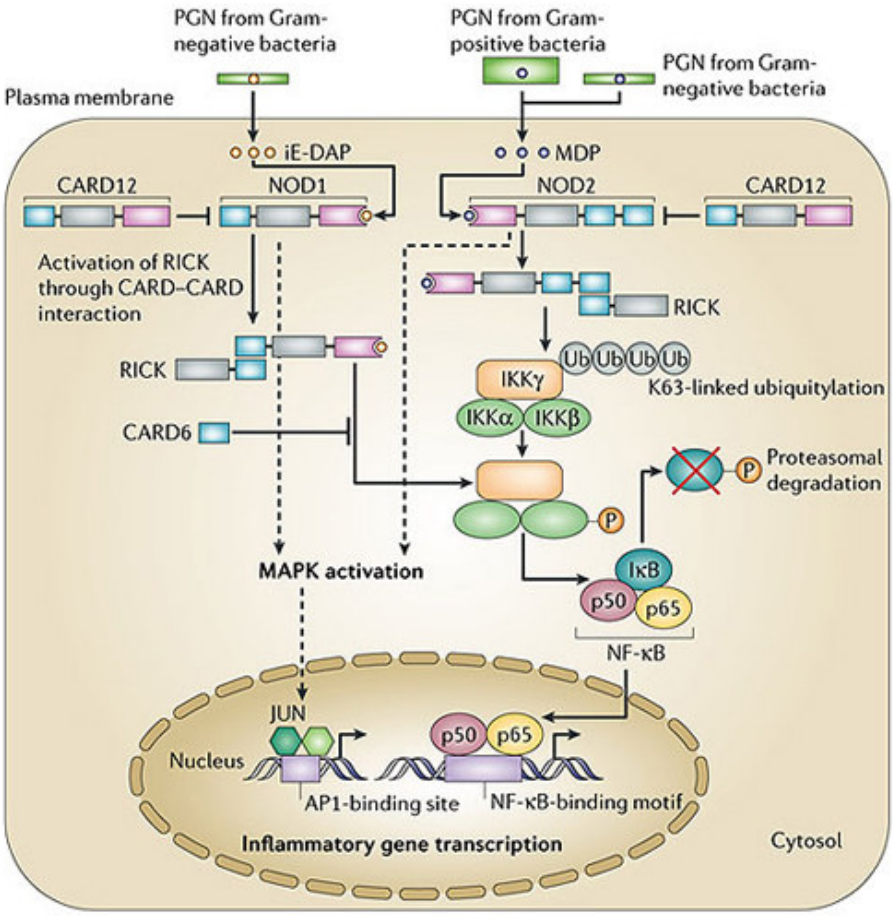

Fig. 2 Signaling pathway of Nod1 and Nod2.

1.1.3 Aim of the study

The septic state, in addition to eliciting a significant inflammatory response, paradoxically renders the host immunocompromised, thereby resulting in increased susceptibility to secondary infections. Thus, we wanted to investigate the effect of the co-administration of Gram positive and Gram negative bacteria on the release of CXCL-8, principal neutrophil chemoattractant, on human lung epithelial cells.

1.2 Materials and Methods

1.2.1 Culture and preparation of bacteria.

Clinical blood culture isolated of *S.aureus* H380 and *E.Coli* 0111.B4 were stored frozen in 15% glycerol. To culture, they were first streaked onto agar plates from which single colonies were inoculated into RPMI-1640 with 10% fetal bovine serum (FBS) and glutamine. Cultures were incubated at 37^0 C overnight and then centrifuged at 800g to pellet the bacteria. Bacteria were washed twice, and re-suspended, in sterile saline. Aliquots of the bacterial suspension were serially diluted and plated onto agar in order to quantify the cell density. The bacterial suspensions were then heat treated for 45 min at 70°C to kill all bacteria; sterility was confirmed by plating of the resultant suspension. Suspensions were adjusted to 10^{10}-10^{12} colony forming units per ml (CFU/ml) and stored frozen at -20ºC in saline containing 15% glycerol.

1.2.2 Cell Culture.

Human lung adenocarcinoma cell line (A549) was cultured in Dulbecco's modified eagles medium (DMEM) containing 10% FBS, L-glutamine (2mM), penicillin (100U/mL), streptomycin (100µg/ml) and MEM non-essential amino acids (1% v/v) in an atmosphere of 5% CO_2 at 37°C. Prior to experimentation cells were seeded into 96-well plates at a concentration of 10^5 cells per well and allowed to

rest for 24 h before stimulation with whole bacteria or selective TLR and Nod ligands for 24 h. Supernatants were used for measurement of CXCL-8 levels. The effect of all the reagents on A549 metabolism was assessed, by measuring the mitochondrial-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Poole, UK) to formazan. This was performed following all treatments.

1.2.3 Measurement of Chemokine Production.

CXCL-8 release in cell free supernatant were determined by Enzyme-Linked Immunosorbent Assay (ELISA) using commercially available matched antibody pairs following a protocol furnished by the manufacturers (R & D systems, Oxford, UK). CXCL-8 concentrations were measured at 450 nm with a reference filter at 550 nm and results expressed as pg/ml.

1.2.4 Assessment of barrier integrity.

Cell culture inserts (PET membrane, 3.0micron pore-size; BD Falcon^{TM}, NJ, USA) were used to determine whether bacteria treatments affected the barrier integrity (Fig. 3). 0.2 ml of medium was added on the upper compartment whilst 0.7 ml into the lower compartment, underneath the membrane where the cells were seeded ($2x10^5$ cells/well). These volumes were suggested by the company in order to avoid the pressure impairment between the lower and upper compartment. Horseradish peroxidase (HRP, 1U/ml; Sigma, UK) was added 30 minute after the treatment. Aliquots of 50µl were collected from the lower compartment after 1 hour HRP addition. HRP activity was measured following Sigma pyrogallol enzymatic assay of peroxidase, which used the following reaction:

$$H_2O_2 + \text{Pyrogallol (donor)} \xrightarrow{\text{Peroxidase (HRP)}} 2H_2O + \text{Purpurogallin (oxidized donor)}.$$

After 5 minutes the addition of pyrogallol, the amount of purpurogallin was read at the spectrophotometer at 405nm and related to the amount of HRP present in the solution, able to catalyze the above described reaction. The values are expressed in HRP U/ml as related to a standard curve of HRP 0.007-0.5 U/ml, and extrapolated using a linear regression analysis from GraphPad Prism 4.0 program.

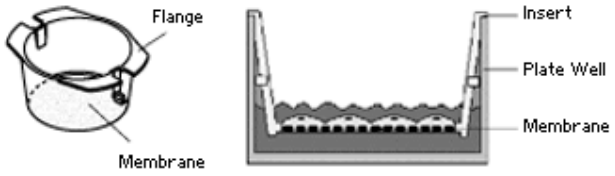

Fig. 3 Cell culture inserts used for the assessment of the barrier integrity

1.2.5 Reverse Polymerase Transcriptase and Real-Time Chain Reaction.

The cells were seeded at $1x10^6$/well in a 6-well plate and serum deprived for 24 hours. Total RNA was isolated from the cells after stimulation using RNeasy minikit (Qiagen Ltd, UK). cDNA was generated by reverse transcription using random primers (Promega) at a concentration of 125 µg/ml. The cDNA was used as a template in the subsequent polymerase chain reaction (PCR) analyses. Transcript levels were determined by real time PCR (Rotor Gene 6; Corbett Research, Sydney, Australia) using the Taqman Universal PCR Master Mix Reagent Kit (Applied Biosystem, UK) and commercially available primers for CXCL8 (Hs00174103_m1; Applied Biosciences, CA, USA). PCR conditions were as follows: step 1, 10 min at 95oC; step 2, 15s at 95 ºC; step 3, 60s at 60 ºC and repeated for 40 cycles. Data from the reaction were collected and analysed (Corbett Research, Sydney, Australia) and relative quantifications of gene expression were calculated using standard curves and normalized to GAPDH.

1.2.6 Statistical Analysis.

Prism 4.0c (GraphPad, inc) was used for all statistical analyses All data shown is mean ± SEM. Statistical analysis was preformed using a One-Way ANOVA or one-sample T-test. Values of P less than or equal to 0.05 were considered to be significant.

1.3 Results

1.3.1 Effect of the whole bacteria on the human lung epithelial cells.

We treated A549 cells with *Staphylococcus aureus,* Gram positive bacteria, and *Escherichia Coli,* Gram negative bacteria, from the concentration 10^5 to 10^8 CFU/ml. As expected, either S.*aureus* or E.*coli* released CXCL-8 in a concentration-dependent manner (Fig 3A and B) but S.*aureus* promoted a markedly reduced release of the chemokine compared to the Gram negative bacteria at 24 hours. Though, the level of the chemokine tended to increase at 48 and 72 hours. Similarly to the protein level, mRNA for CXCL-8 was lower expressed after stimulation with S.*aureus* despite E.*coli* (Fig 3C) at 3 hours. Interestingly, the co-administration of both bacteria at the highest concentration (10^8CFU/ml) induced a more than additive effect on CXCL-8 detection either in terms of protein or mRNA (Fig 3C and D).

1.3.2 Effect of the bacteria on the lung epithelial barrier integrity.

As the type II epithelial cells also function as a barrier in the lung, we tried to investigate whether the co-administration of the bacteria could affect the physiological epithelial barrier integrity. We measured HRP levels in the supernatant recovered from the lower department of the cell culture insert after 1 hour the enzyme addition. The amount (HRP U/ml) detected was a measure of enzyme crossed through the layer of cells lying on the membrane of the insert. Surprisingly, we detected a higher amount of enzyme after S.*aureus* despite E.*coli* treatment (Fig 4A), whilst the co-administration of both bacteria did not increase HRP levels. In order to understand whether the barrier disruption could be ascribed to the CXCL-8 production, we treated the cells with increasing concentration of CXCL-8 (3-30-300 ng/ml, R&D System, CA, USA) (Fig 4B) and its relative

antibody (Ab) (4µg/ml, R&D System, CA, USA). The higher doses (30-300ng/ml) of the chemokine did not increase HRP amount in the supernatant as well as for E.*coli* and E.*coli*+S.*aureus* treatment. Furthermore, the administration of the antibody reverted in a significant manner HRP amount detected after either CXCL-8 (3ng/ml) or S.*aureus* treatment (Fig. 4C). Besides, E.coli reduced the amount of HRP detected in the lower compartment of the insert (Fig. 4D) in a significant manner ($P<0.0001$).

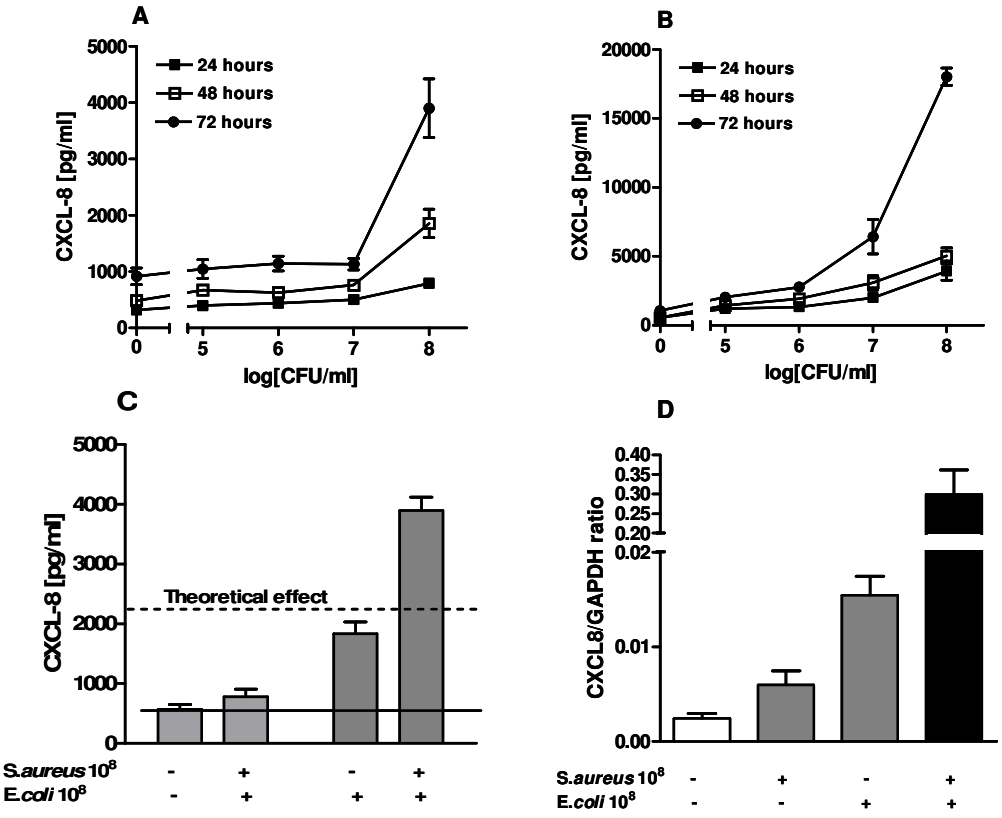

Fig.3. Effect of bacteria on CXCL-8 production from A549. The cells were treated with S.*aureus* A) and E.*coli* B) in a cumulative concentration manner at 24, 48 and 72 hours. C) Co-administration of both bacteria revealed a more than the theoretical additive effect on CXCL-8 production, confirmed by the D) RT-PCR. The theoretical effect is the addition of the effect of the bacteria alone.

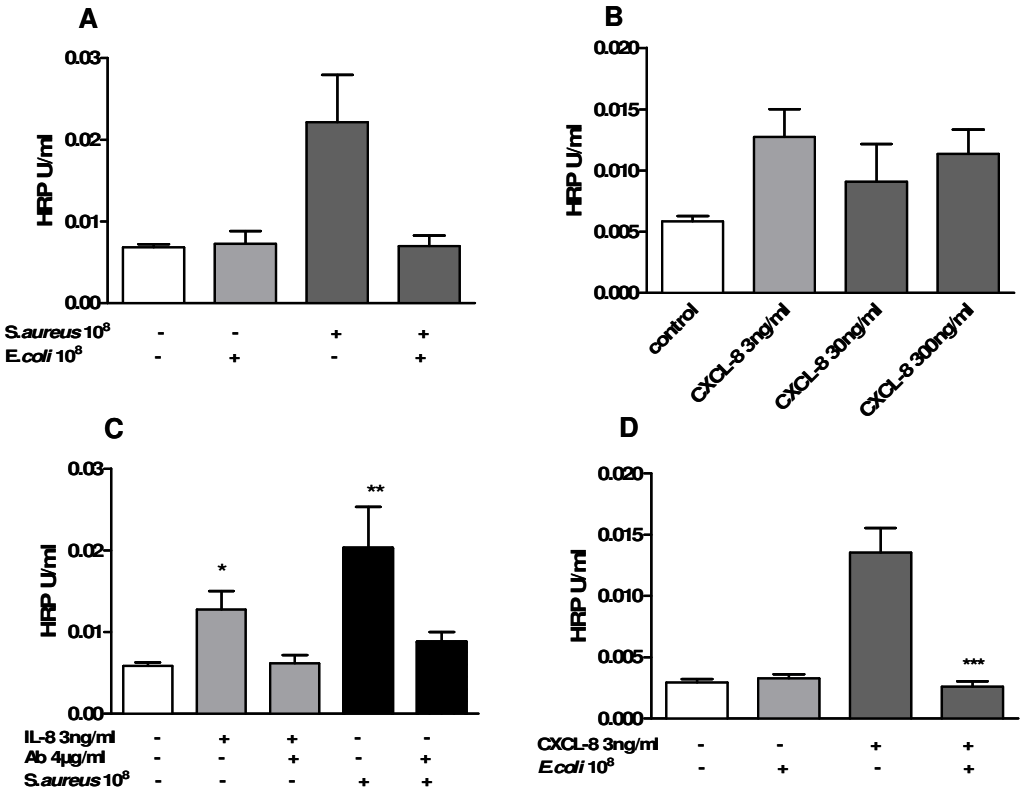

Fig. 4 A) Effect of the bacteria and their combination on the barrier integrity B) The administration of CXCL-8 did not show a concentration-dependent disruption of the barrier integrity. C) The Ab for CXCL-8 significantly reverted the effect of CXCL-8 (*P<0.05) and S.*aureus* on the barrier integrity (**P<0.001). D) E.*coli* reduced CXCL-8 effect in a significant manner (P<0.0001). One way Anova multiple comparison Bonferroni's post test.

1.3.3 Effect of TLR2 and Nod2 ligands on the human lung epithelial cells.

In order to mimic all the Gram positive cell wall components known to activate TLRs and Nods protein (4), we used Pam3CSK4 (100ng/ml) as a TLR2/1 ligand, FSL-1 (100ng/ml) as a TLR2/6 ligand and MDPLys18 (1μM) as a Nod2 ligand. The co-administration of all the above mentioned ligands *plus* E.*coli* revealed an additive effect on CXCL-8 production (Fig 5E). Noticeably, the administration of Pam3CSK4 and MDPLys18 with the bacteria revealed a significant ($P<0.0005$, $P<0.001$, respectively) reduced actual effect despite what expected (Fig 5B and C) (Table I), whilst FSL-1+E.*coli* and FSL-1+Pam3CSK4+E.*coli* induced an additive release of CXCL-8 (Fig 5A and D). Besides, FSL-1+Pam3CSK4 alone confirmed the addition (Fig 5B, C and D) (Table I) and furthermore, FSL-1+Pam3CSK4+MDP revealed a significant induction ($P<0.01$) on CXCL-8 release compared to the theoretical additive effect. So, even though TLR2/1 and Nod2 co-activity tended to contrast the chemokine release, the concomitant activation of TLR2/6 *plus* the PRRs from the whole Gram negative bacteria cell wall component tended to counteract the inhibitory effect.

1.3.4 Effect of TLR4 and Nod1-2 ligands on the human lung epithelial cells.

In the same way, we tried to mimic the Gram negative cell wall components by using LPS (1μg/ml), which activates TLR4, and FK565 (1μM) as Nod1 ligand and MDPLys18. We used both Nod ligands, MDPLys18 and FK565, as it is well known that the PGN from the Gram negative bacteria can activate both proteins (4). Interestingly, MDPLys18+S.*aureus* or FK565+S.*aureus* induced an additive effect on CXCL-8 release, but singularly co-administered with LPS without the bacteria did not have the same effect (Fig 6A, B, D and E) (Table II). The co-administration of LPS+MDPLys18 and the further addition of FK565 without the bacteria revealed

a marked reduction of CXCL-8 release ($P<0.005$ and $P<0.0001$, respectively), compared to the theoretical additive effect of these chemicals alone (Table II). Another interesting result was the amount of CXCL-8 detectable after the stimulation with LPS+S.*aureus* (Table II). The latter treatment revealed a synergistic effect similar to the effect observed by the co-administration of the whole both bacteria. By contrast, the combination of all the above mentioned ligands with S.*aureus* induced a reduced amount of CXCL-8 than expected ($P<0.01$) (Fig. 6F).

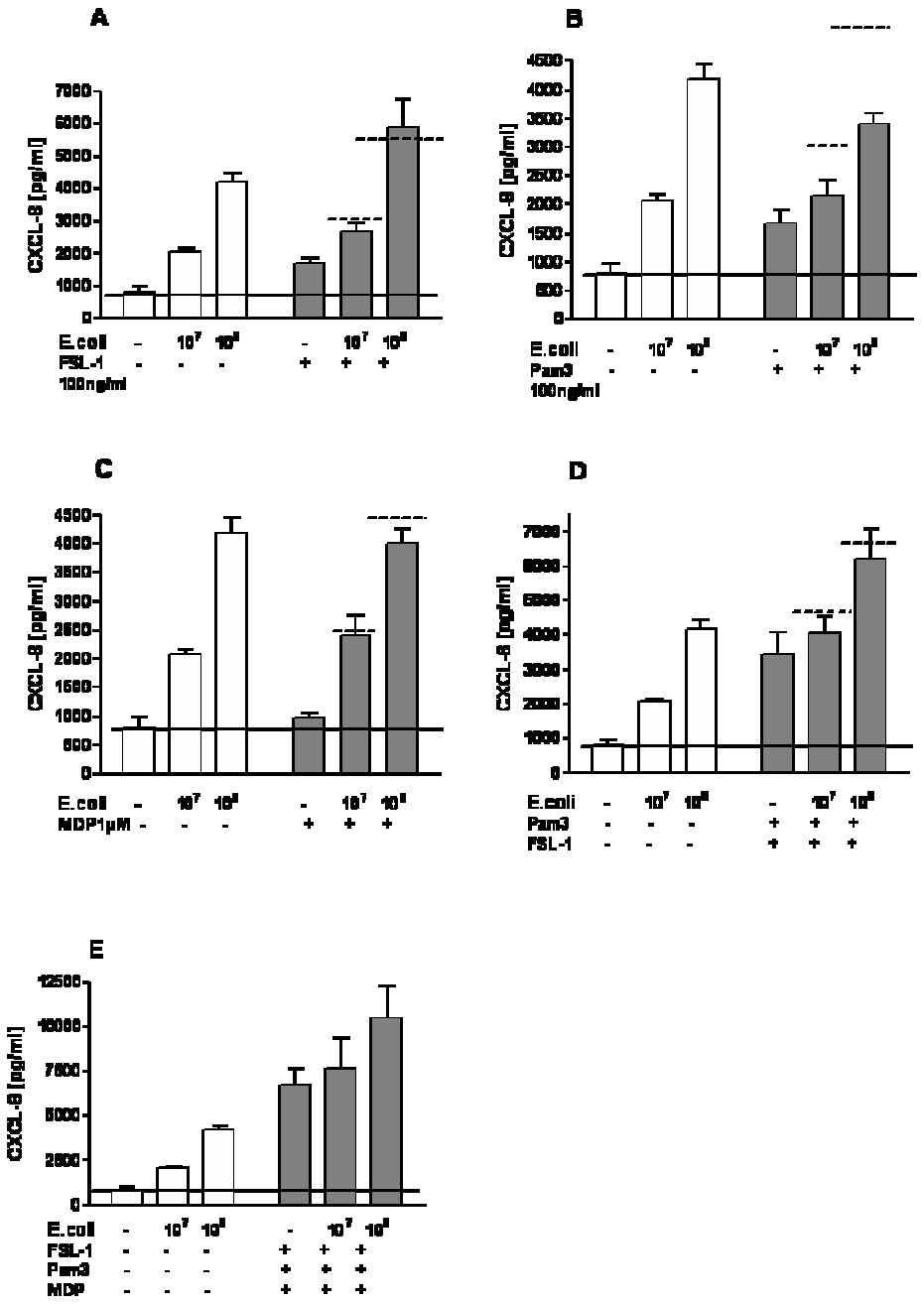

Fig. 5 Effect of PAMPs+E.*coli* on CXCL-8 release. A) FSL-1 gave an additive effect on CXCL-8 release; B) Pam and C) MDP combined with E.*coli* released less than an additive amount of CXCL-8; D) FSL-1+Pam+E.*coli* showed an additive release of CXCL-8; E)The addition of the above PAMPs together revealed an additive release of CXCL-8. The non continuous lines correspond to the predicted additive effect, and the continuous line corresponds to the effect except the basal.

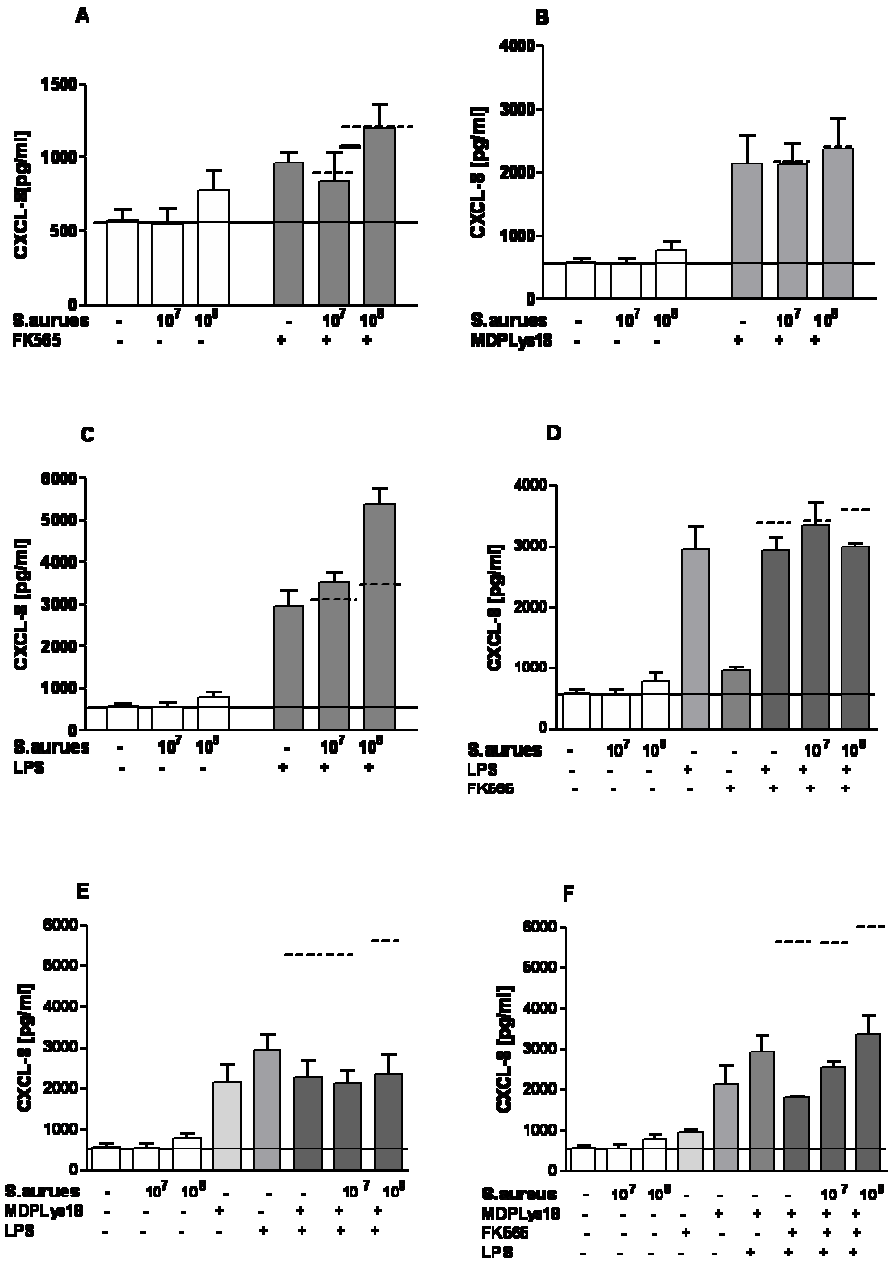

Fig. 6 Effect of PAMPs+S.*aureus* on CXCL-8 release. A) FK565 as well as B) MDPLys revealed and additive effect on CXCL-8 production; C) LPS+S.*aureus* induced a synergistic effect but combined with D) FK565 or E) MDP released less than an additive amount of CXCL-8; F) the combination of the above PAMPs reduced significantly CXCL-8 production. The non continuous lines correspond to the predicted additive effect, and the continuous line corresponds to the effect except the basal.

Table I. Effect of possible PAMPs combination on CXCL-8 release (pg/ml) after E.*coli* administration. ***P<0.0005, **P<0.01, *P<0.05 *vs* actual effect (One sample T test).

		Single effec	Treatment	Actual effe	Theoretic effect
E.coli 108		3388.4±266.	**E.coli+Pam3CSK4**	2598.9±197	4257.3***
Pam3CSK4 100ng/ml		886.9±221.9	**E.coli+FSL-1**	5061.7±875	4282
FSL-1 100ng/ml		893.9±164	**E.coli+MDP**	3214.6±254	4948**
MDP 1µM		1559.03±469	**E.coli+Pam+FSL-1**	5388.3±907	5169
			E.coli+Pam+FSL-1+MDP	9608±1801	6728
			MDP+Pam3CSK4+FSL-1	5907.5±953	3339.8*
			FSL-1+Pam3CSK4	2603±690.6	1780

Table II. Effect of possible PAMPs combination on CXCL-8 release (pg/ml) after S.*aureus* administration. ****P<0.0001, ***P<0.005, **P<0.01, *P<0.05 *vs* actual effect (One sample T test).

		Single effec	Treatment	Actual effe	Theoretic effect
S.aureus 108		203.82±133.	**S.aureus+LPS**	4788.1±392	2577.9***
LPS 1µg/ml		2374.15±381	**S.aureus+FK565**	626.5±159.	593
FK565 1µM		389.4±67.3	**S.aureus+MDP**	1803±469	1762
MDP 1µM		1559.03±469	**S.aureus+LPS+FK565**	2415.7±74.	2967.3*
			S.aureus+LPS+FK565+MI	2782.8±479	4526.25**
			S.aureus+LPS+MDP	3424.4±682	4136
			LPS+FK565	2362.4±213	2763.
			LPS+FK565+MDP	1241.1±36.	4322.6****
			LPS+MDP	1698.4±413	3933.2***

1.4 Discussion

Neutrophils are the first immune cells that arrive at the site of infection. They are recruited by the production of CXCL-8, which facilitates the insertion in the infected tissue. Our study is the first report focused on the production of CXCL-8 after whole Gram positive and negative bacteria challenge on human lung epithelial cells.

S.*aureus* stimulation did not induce a relevant increase of chemokine production compared to the effect of E.*coli* at 24hours, even though the amount of CXCL-8 was highly detectable at 48 and 72 hours in a concentration-dependent manner. Interestingly, the co-administration of whole both bacteria induced a synergistic effect at 24 hours in terms of protein and mRNA quantity. The involvement of E.*coli* in sepsis is well-established, but there is an increasing evidence for S.*aureus* implication (1). Beyond the organ where sepsis originates, the lung is usually the first to fail, in part due to the rapid accumulation of neutrophils in the narrow lumen of lung capillaries. Therefore, we analysed the effect of the combination of both bacteria on the lung epithelium integrity. Surprisingly, the co-administration of both bacteria did not disrupt the epithelial barrier, despite S.*aureus* alone which was able to increase the amount of peroxidase detectable in the lower department of the inserts used. This effect was related to CXCL-8 as demonstrated by the use of CXCL-8 antibody, which reverted the effect of S.*aureus* on the barrier damage, and further confirmed by the co-addition of E.*coli* with CXCL-8, obviously for an extreme amount of CXCL-8 produced. Blease et al. (15) demonstrated a bell shaped release of CXCL-8 after S.*aureus* LTA *plus* LPS stimulation on human airway microvascular endothelial cells. The results indicated that the percent of the adhesion of neutrophils was less than predicted for an additive effect. Besides, in a

mouse model of S.*aureus*-induced arthritis, in which either wild type and CAM deficient mice were compared, the leucocyte-mediated damage was reduced in CAM KO mice, even though the mortality was greater in these mice than the control, according to the fewer leucocytes counted in the injured tissue (15). This suggests that a high recruitment of leucocytes could be essential for protection against systemic disease but it may exacerbate local manifestations. In the same way, our results showed a less damage from the co-administration of both bacteria on the barrier integrity than S.*aureus* alone. So, the Gram positive bacteria did not induce a great amount of chemokine but a greater leakage of HRP, confirming the extreme damage that S.*aureus* could induce in a condition of septic shock.

We also tried to investigate the PRRs involved in the synergy between S.*aureus* and E.*coli* on CXCL-8 release. Therefore, we used single or combined PAMPs added to the whole Gram positive or Gram negative bacteria, respectively. Interestingly, the addition of ligands that mimicked for S.*aureus* cell wall components *plus* E.*coli* revealed an additive response on CXCL-8 whilst the addition of all E.*coli* cell wall components with S.*aureus* revealed a remarked reduction. By contrast, S.*aureus*+LPS induced a synergistic release of CXCL-8 but the addition of MDPLys18 induced an opposite effect, further reduced with FK565 administration. So, Nods activation may be responsible of the reduced CXCL-8 production after Gram positive stimulation despite Gram negative bacteria. A recent study assessed the effect of LPS on Nod1 and Nod2 expression in murine macrophages (16). The stimulation with LPS increased the expression of these proteins via TLR4 activation as the antibody for TLR4 reduced Nods expression. According to this study, Nod1 and Nod2 expression may be induced either by NF-kB activation directly, or by the production in a later phase of TNFα, which can act

in an autocrine manner. Furthermore, Watanabe et al. (17) demonstrated that Nod2 can inhibit TLR2-dependent NF-kB activation following MDP and Pam3CSK4 stimulation in an *in vivo* mouse model. These latter studies could explain our results as the activation of TLR4 by E.*coli* could induce Nods expression, leading to an increased release of CXCL-8; on the other side, the activation of TLR2 could be responsible of the reduced effect on CXCL-8 via Nods expression inhibition after S.*aureus* treatment. Our experiments revealed that the administration of E.*coli* with the TLR2/1 and TLR2/6 and Nod2 synthetic ligands induced an additive effect, whereas in the second part of this study we showed that the addition of Nods ligands reduced the effect of LPS on S.*aureus*.

In contrast to our results, other studies on dendritic cells (18) and human monocytes THP-1 [16] suggested that Nod1 and Nod2 could synergize with TLRs agonist in CXCL-8 production. These last observations may be related to the immune nature of the cells considered.

In conclusion, we could speculate that either TLR antagonist or Nods agonist could be used for the future septic shock therapy, in order to reduce the destructive effect of excessive CXCL-8 released, inducing to a protective mechanism for the lung epithelial cells which would be otherwise overwhelmed by neutrophils and their antibacterial products.

References

1. Opal SM, Cohen J. Clinical gram-positive sepsis: does it fundamentally differ from gram-negative bacterial sepsis. *Crit. Care Med*. 1999, 27(8): 1608-16

2. Netea MG, Van der Meer JW, Kullberg BJ. Recognition of pathogenic microorganisms by Toll-like receptors. *Drugs Today (Barc)* 2006; 42 Suppl A: 99-105

3. Jiang ZH, Koganty RR. Synthetic vaccines: the role of adjuvants in immune targeting. *Curr Med Chem* 2003, 10(15): 1423-39

4. O'Neill LA. How Toll-like receptors signal: what we know and what we don't know. *Curr Opin Immunol* 2006, 18(1): 3-9

5. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and immunity. *Nat. Immunol* 2001, 2: 675-80

6. A. Uehara1, S. Yang, Y. Fujimoto, K. Fukase, S. Kusumoto, K. Shibata, S. Sugawara and H. Takada. (2005) **7**(1):53-61

7. Petti CA, Fowler VG Jr. *Cardiol Clin*. 2003, 21(2): 219-33

8. O'Neill LA. Signal transduction pathways activated by the IL-1 receptor/Toll-like receptor superfamily. *Curr Top Microbiol Immunol* 2002, 270: 47-61

9. Brown MA, Jones WK. NF-kappaB action in sepsis: the innate immune system and the heart. *Front Biosc*. 2004, 1:9: 1201-17

10. Akira S. Toll-like receptors: lessons from knockout mice *Biochem Soc Trans* 2000, 28(5): 551-6

11. Khou J, An H, Xu H, Liu S, Cao X. Heat shock up-regulates expression of Toll-like receptor and Toll-like receptor-4 in human monocytes via p38 kinase pathway. *Immunology* 2005, 114(4): 522-30

12. Y. Ogura, N. Inohara, A. Benito, F.F. Chen, S. Yamaoka and G. Nunez, Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-κB, *J Biol Chem* **276** (2001), pp. 4812–4818.

13. S.E. Girardin, I.G. Boneca, L.A. Carneiro, A. Antignac, M. Jehanno, J. Viala, K. Tedin, M.K. Taha, A. Labigne and U. Zahringer *et al.*, Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan, *Science* **300** (2003), pp. 1584–1587

14. N. Inohara, Y. Ogura, A. Fontalba, O. Gutierrez, F. Pons, J. Crespo, K. Fukase, S. Inamura, S. Kusumoto and M. Hashimoto *et al.*, Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease, *J Biol Chem* **278** (2003), pp. 5509–5512.

15. Blease K. Chen Y. Hellewell P, Burke-Gaffney A. J Immunol 1999, 163(11): 6139-47

16 Verdrengh M, Springer T A, Gutierrez-Ramos J C, Tarkowski A. *Infect Immunol* 1996, 64: 2807

17 Takahaski Y, Isuzugawa K, Murase Y, Imai M, Yamamoto S, Iizuka M, Akira S, Bahr G M, Momotani E, Hori M, Ozaki H, Imakawa K. *Biochemistry* 2006, 471-478

18 Watanabe T, Kitani A, Murray P J, Strober W. *Nature Immunol* 2004, 5(8) 800-808

Chapter 2

Differential effect of Gram positive and Gram negative bacteria on macrophages and vascular smooth muscle cells: role of TLRs

2.1 Introduction

Septic shock is a medical condition that promotes a massive vascular hypotension with a consequent circulation failure that impairs blood and oxygen providing to the tissues. In clinical practice, the key symptom is a severe fall in blood pressure, which is often associated with the dysfunction or failure of several important organs, like lung, kidney, liver and brain. The most common cause of shock is the infection of blood with bacteria resulting in systemic infection and ultimately septic shock.

Current therapeutic approaches for septic shock are the use of antimicrobial chemotherapy, glucocorticoids, volume replacement, inotropic and vasopressor support, oxygen therapy; even though a high estimated mortality is still ranging from 50% and 80% (1).

Several studies revealed that the key biological mediator for profound hypotension and hyporesponsiveness to vasoconstrictors in the septic shock was nitric oxide (NO) (1).

2.1.1 Nitric oxide

NO is a gas with pleiotropic functions in a wide variety of physiological systems. In the cardiovascular system, NO exerts vasodilatory, antiadhesive and antiproliferative effects.

NO is generated from L-arginine by a family of enzymes called nitric oxide synthase (NOS). The oxidation of one of the guanidino nitrogen atoms of this semiessential aminoacid by NOS is associated with the oxidation of NADPH and the reduction of molecular oxygen. Thus, NOS contains an oxygenase domain, which has the catalytic site, and a reductase domain. The oxygenase domain appears to contain binding sites for tetrahydropterin (BH4), heme and L-arginine.

The synthesis of NO from L-arginine and molecular oxygen involves the following: the generation of N^G- hydroxy-L-arginine and water (first step), and subsequently the oxidation of N^G- hydroxy-L-arginine in the presence of molecular oxygen to form NO, L-citrulline and water (second step).

NO is generated by many mammalians cells by three isoforms of NOS, NOS I, NOS III and NOS II. NOS III, also called eNOS, is expressed in endothelial cells, whilst NOS I, also called nNOS, is present at the level of neuronal cells (2). NOS I and NOS III are constitutively expressed and both enzymes require calcium for their activation. NOS II, also called iNOS, is an inducible isoform, functionally independent of intracellular changes of calcium, and activated through the nuclear factor NF-kB, which in turn is activated during an inflamed *status*. In contrast to the constitutive isoforms, iNOS is synthesized *de novo* during inflammation and produces large amounts of NO over prolonged periods of time (3).

The production of NO by the endothelial cells through eNOS, leads to the transmigration of this gas through the cell membrane of vascular smooth muscle. NO binds the soluble guanilate cyclase (sGC), leading to the synthesis of the cyclic guanosine-monophosphate (cGMP), which then activates the cGMP dependent protein kinase that phosphorilates the light chain of the myosin, inducing the vasodilation (Fig.1) (4).

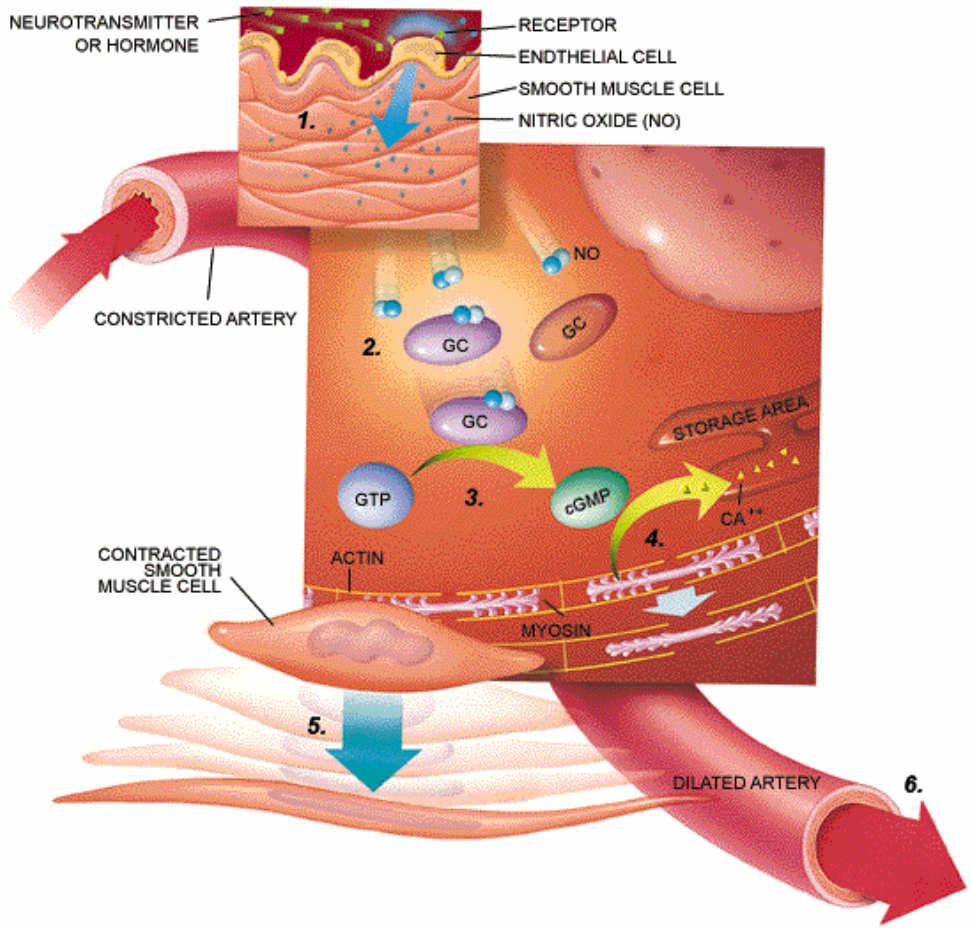

Fig.1 A stimulus, like acethylcoline, can react on its own receptor on the endothelial cell and transducer for the NO-dependent pathway (1). NO diffuses through the smooth muscle cell and activate the sGC (2) with a consequent release of cGMP (3), which in turn, in concomitance with intracellular calcium, activates the PKG kinase (4). PKG phosphorilates the light chain of the myosin (5), inducing vasodilation (6).

2.1.2 Role of NO in the septic shock

Several studies have supported that the endogenous large amount of NO was responsible of the systemic hypotension, organ failure and vascular hyporesponsiveness to vasoconstrictors, typical of the septic shock. This is evidenced by the increased levels of nitrite (NO^{2-}) and nitrate (NO^{3-}, stable metabolite of NO) measured in the plasma of septic patients, inflammation-induced iNOS expression and by the ability of selective iNOS inhibitors to restore blood pressure in experimental models of sepsis and reverse hypotension in human endotoxaemia (5).

iNOS is effectively absent under physiological conditions but it is expressed in many cell types in response to pro-inflammatory cytokines and lipopolysaccharide (LPS). In fact, NO can be produced by both stromal cells, like endothelial, vascular smooth muscle and epithelial cells, and by immunocompetent cell, like macrophages. The local generation of large amount of NO by activated macrophages serves as host defence, capable of killing the bacteria through the 'suicide mechanism', which implicates either a cytostatic or cytotoxic effect (6). Indeed, large amount of NO cause autoinhibition of the mitochondrial respiration by inhibiting several key enzymes in the mitochondrial respiratory chain, like NADH-ubiquinone reductase or succinate-ubiquitinone oxidoreductase, and the Krebs' cycle, through the formation of radical species (7). Besides, NO, like other radicals and oxidants, causes damage at the double strand of DNA that triggers an energy-consuming repair cycle by activating the nuclear enzyme poly(ADP)ribosyltransferase (PARP). Activation of PARP results in the rapid depletion of intracellular NAD+, its substrate, slowing the rate of glycolysis, electron transfer and ATP formation, which ultimately leads to cells death (8).

These effects can be ambiguous. They can be traduced onto the guest or onto the host organism; on one side, the immune system is activated to combat the invasion of the pathogen, but on the other side the same host organism can be injured, as exemplified by the cardiovascular system dysfunction during the sepsis (9).

The induction of iNOS by the endotoxin, like LPS, the most used bacterial component for the experimental model of sepsis *in vivo* and *in vitro,* is secondary to the release of the proinflammatory cytokines like IL-1, TNFα and interferon IFN-γ, which alone or in concert, with the platelet-activating factor (PAF) activate cells to express iNOS protein and activity (10).

In several studies it has been demonstrated the involvement of Toll-like receptors (TLRs) implicated in the pathology of septic shock (11). TLRs are able to recognise pathogens and in particular pathogen-associated molecular patterns (PAMPs), which are ligands for pattern recognition receptors (PRRs). Gram negative bacteria activate TLR4 whilst Gram positive bacteria are recognised by TLR2 (12). The activation of TLRs has been considered really important in the first host immune defence line.

2.1.3 Aim of the study

The aim of this study was to investigate the involvement of macrophages in NO production after bacteria challenge. We wanted to evaluate a comparison between two different cell types like macrophages, representing the immunocompetent cells, and vascular smooth muscle cells, representing stromal cells. In particular, we focalised on the involvement of TLRs.

2.2 Materials and methods

2.2.1 Culture and preparation of bacteria.

Bacteria were prepared in the same way as described in the paragraph 1.2.1.

2.2.2 Cell culture.

Cultured cells murine macrophages (J774.2) or rat aortic vascular smooth muscle cells (RASMs) were cultured in Dulbecco's modified eagles medium (DMEM) containing 10% FBS, penicillin (100U/mL), streptomycin (100µg/ml), and MEM non-essential amino acids (1% v/v) in an atmosphere of 5% CO2 at 37°C. The medium was refreshed every 48 hours or as required. The cells were plated in a 96-well plate at 70-80% of confluence and left for 24 hours before measuring the amount of nitrite into the supernatant.

2.2.3 Bone marrow derived macrophages

Primary macrophages were cultured from the bone marrow of the legs from wild type C57BL/6, TLR4 knock out mice. $TLR4^{-/-}$ mice were kindly provided by S. Akira (Osaka University, Osaka). All strains were backcrossed for five generations into the C57BL/6 strain. Wild type C57BL/6 mice were used as controls. All experiments were performed using male mice, aged 10-14 weeks.

The legs were removed from sacrificed mice and cleaned of muscle and connective tissue. The bones were cut at both ends and bone marrow was flushed out using a 1 ml syringe and 19 gauge needles. Bone marrow derived cells were placed into RPMI 1640 medium containing 1mM sodium pyruvate, L-Glutamine and phenol red, supplemented with penicillin 100U/ml, streptomycin (100µg/ml), L-Glutamine 2mM, 10% foetal calf serum, 5% horse serum and 2-Mercaptoethanol.

The suspension of bone marrow was spun with an excess of medium at 200g for 5minutes. The resulting pellet was resuspended in fresh complete medium supplemented with M-CSF (Sigma, UK) and separated into 4 Petri dishes for 3

days. At day 4, the media was removed and replaced and left incubated for further 2 days at 37ºC, 5%CO_2 and 95% air. At day 7, the cells were used.

2.2.4 Measurement of NO.

Nitric oxide was measured by the formation of nitrite in culture medium using the Griess reaction. We used the Griess reagent 1, composed of 10g of sulphanilamide (Sigma, UK) added to 950 ml of distilled water, and the Griess reagent 2, composed of 5g of napthylethyl-endiamine dihydrochloride (Sigma, UK) added to 1L of distilled water. The two reagents, previously let equilibrate for 15 minutes, were added to 100 µl of samples and standard curve (Sodium Nitrite-Sigma-0-0.5mM). Sulphanilamide reacted with the nitrite and the ethylendiamine in the solution, giving a final product characterized by an azo-group, able to give a purple color. The reading wavelength was at 550nm.

2.2.5 Measurement of NOSII expression

NOSII expression in primary rat aortic smooth muscle cells was measured by Western Blotting. In brief, cells were plated into 6-well culture plates and treated with *S.aureus* or *E.coli* for 48 h. The medium was removed and the cells were washed twice with ice cold PBS. Cells were lysed using HEPES (10mM) containing $MgCl_2$ (3mM), KCl (40mM), Glycerol (5%), Nonidet P-40 (0.3%), PMSF (1mM). Protein concentration in whole cell preparations was measured using the Bradford assay. Samples were separated by gel electrophoresis on 8% SDS-polyacrylamide gels; after transfer onto nitrocellulose membranes NOSII was detected using specific polyclonal rabbit antibody (1:1000; SC-650; Santa Cruz Biotechnology, CA, USA) and the signal amplified with a goat anti-rabbit IgG-horseradish peroxidase (1:1000, DakoCytomation, Cambridge, UK). Blots were visualised on to film using ECL reagents (Amersham Biotechnology, Oxford, UK). After the blots were stripped using Restore Western Blot Stripping Buffer (Pierce, IL, USA), the

blots were probed with α-tubulin (1:2000; Abcam, Cambridge, UK) that was used to confirm equal protein loading between lanes.

2.2.6 Reverse Polymerase Transcriptase and Real-Time Chain Reaction.

Total RNA was isolated from murine aortae or J774 cells using the RNeasy Mini Kit (QIAGEN, Crawley, UK), after stimulation for 3 hours. cDNA was generated by reverse transcription using random hexamers. The cDNA (5µg/reaction) was used as a template in the subsequent polymerase chain reaction (PCR) analyses. Transcript levels were determined by real-time PCR (Rotor Gene 3000; Corbett Research, Sydney, Australia) using the Sybr Green PCR Master Mix Reagent Kit (Promega, Madison, WI). The sequences of PCR primers were:

TLR2: sense, 5'-GCCACCATTTCCACGGACT;

antisense, 5'-GGCTTCCTCTTGGCCTGG;

TLR4: sense, 5'-AGAAATTCCTGCAGTGGGTCA;

antisense, 5' TCTCTACAGCTGTTGCTTGCACATGTCA;

TLR 1: sense, 5'-TTGGCAACATGTCCCAACTA;

antisense, 5'-ATGAGCAATCAGCTGCACAC;

TLR 6, sense, 5'-CAAAGGAGGCGCTATACTCG;

antisense, 5'-GCACACCATGTGGATGAAAG;

GAPDH: sense, 5'-TCCACGACATACTCAGCAC;

antisense, 5'-AACGACCCCTTCATTGAC.

Primers were used at a concentration of 1 µM for real time. Cycling conditions for real-time PCR (a total of 60 cycles used) were as follows: step 1, 15 min at 95°C; step 2, 25s at 65°C (TLR2) or 60°C (TLR4, TLR1, TLR6) or 55°C (GAPDH), 25s at 72°C; step 3, 5min at 72°C, step 4, 5s at 65°C to 95°C. Data from the reaction were collected and analyzed by the complementary computer software (Corbett

Research). Relative quantifications of gene expression were calculated using standard curves and were normalized to GAPDH.

2.2.7 Measurement of Cytokine Production.

TNFα release in cell free supernatant were determined by Enzyme-Linked Immunosorbent Assay (ELISA) using commercially available matched antibody pairs following a protocol furnished by the manufacturers (R & D systems, Oxford, UK). TNFα concentrations were measured at 450 nm with a reference filter at 550 nm and results expressed as pg/ml.

2.2.8 Statistical analysis.

Prism 4.0c (GraphPad, inc) was used for all statistical analyses. All values are expressed as mean ± SEM. Two Way Anova or t test were performed to statistically analyse the data. Values of p less than or equal to 0.05 were considered to be significant.

2.3 Results

2.3.1 Production of NO in macrophages

Under basal culture conditions, J774.2 macrophages released low or undectable levels of NO. We stimulated the cells with increasing concentration of E.*coli* (10^7-$3x10^8$CFU/ml), Gram negative bacteria, sensed by TLR4, and S.*aureus* (10^7-$3x10^8$CFU/ml), Gram positive bacteria sensed by TLR2 principally (12). As expected, E.*coli* induced NO release from cultured murine macrophages in a concentration dependent manner (Fig.2). However, when cells were stimulated with S.*aureus*, the amount of NO detected was significantly lower to the one produced by E.*coli* ($P<0.001$, Fig.2).

To evaluate whether the Gram positive bacteria effect was only related to NO, we measured the amount of TNFα released after S.*aureus* administration on the J774.2 macrophages. Surprisingly, S.*aureus* was able to induce TNFα production in a concentration dependent manner as well as E.*coli* (Fig.3). Besides, there was no difference between the two bacteria treatment in this cytokine production.

The implication of TLRs on NO production is already assessed, so we analysed the expression of mRNA for TLR4, TLR2, TLR1 and TLR6 on macrophages. Under basal conditions, murine macrophages expressed relatively low levels of TLR2 and TLR4 compared to TLR1 and TLR6 (Fig.4). TLR4 was not increased after E.*coli* stimulation, even though the basal level was very high, assessing the role of these receptors on immune competent cells (Fig.5). In contrast, TLR2 and especially TLR6 were significantly increased after S.*aureus* (10^8CFU/ml) stimulation ($P< 0.05$, $P<0.001$, respectively) (Fig.6A, B).

As the NO was detectable only after E.*coli* treatment, we used bone marrow TLR4-/- derived macrophages in order to evaluate the role of TLR4. $TLR4^{-/-}$ derived

macrophages were not responsive to E.*coli* (10^{-7}-$2x10^{-8}$) or LPS (0.001-1μg/ml) in terms of NO release compared to the wild type mice derived macrophages (Fig. 7A, B). TLR4 was responsible of NO production after E.*coli* and LPS stimulation.

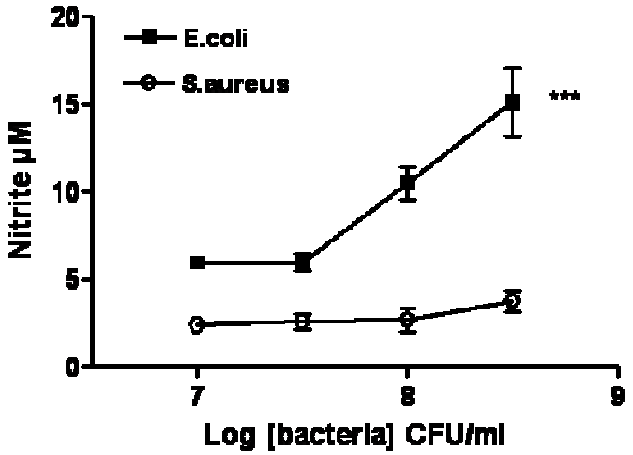

Fig. 2 E.*coli* (10^7-3x10^8 CFU/ml) induced a significant (P<0.001) increase in NO production despite S.*aureus* at 24 hours on J77.2

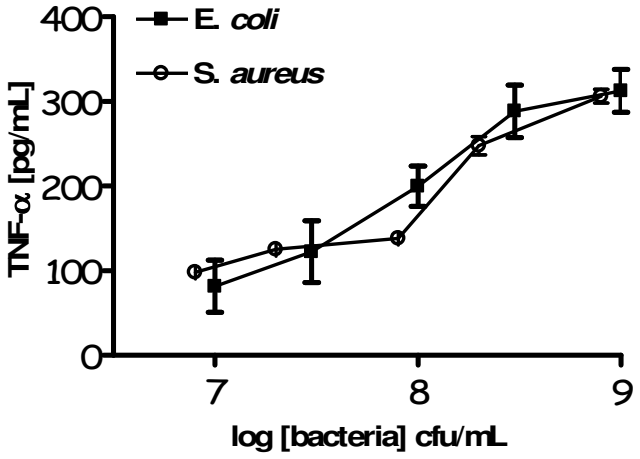

Fig. 3 There was no difference in the production of TNFα on murine macrophages after S.*aureus* or E.*coli* stimulation.

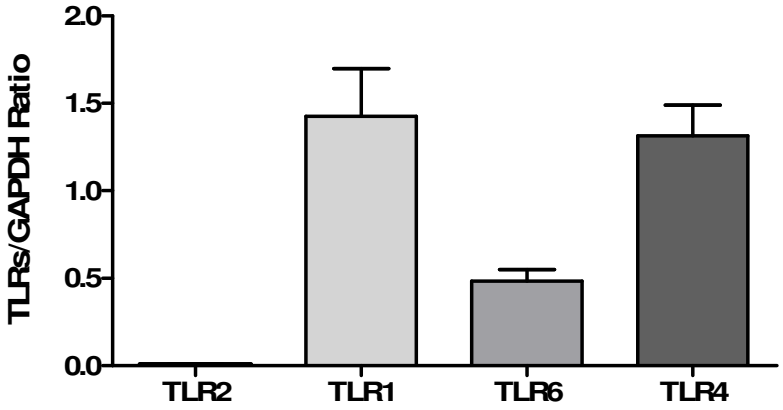

Fig.4 Expression of TLRs on murine macrophages under basal culture conditions.

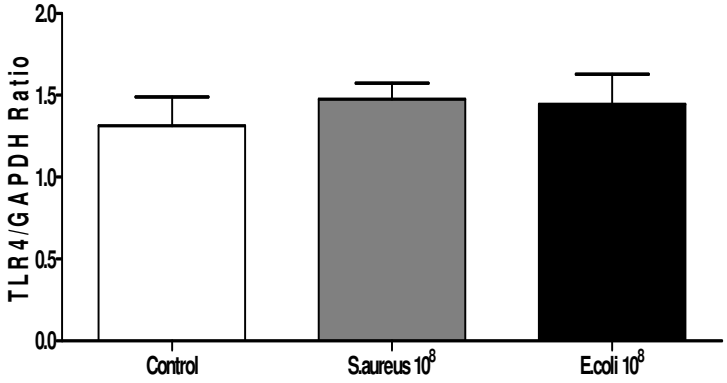

Fig. 5 TLR4 mRNA was not modified after E.*coli* and S.*aureus* stimulation on macrophages.

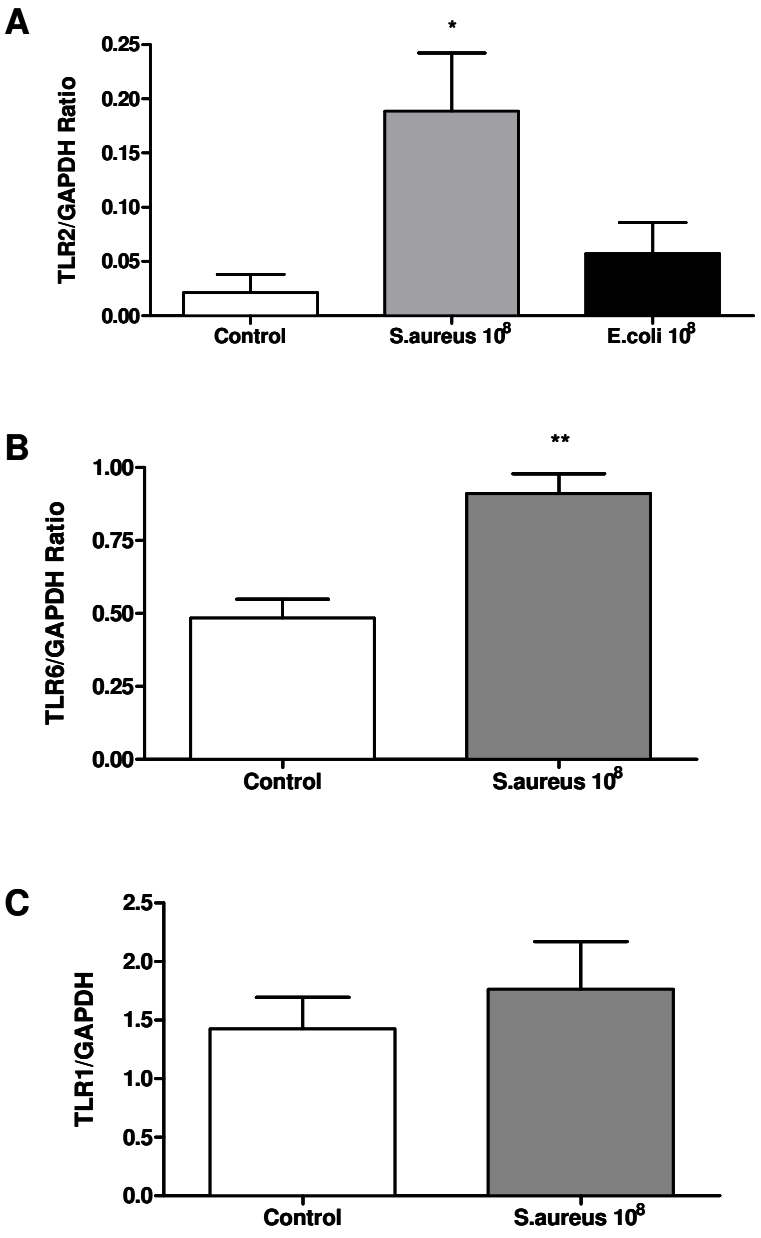

Fig. 6 A) TLR2 mRNA was significantly increased after S.*aureus* stimulation ($P<0.05$); B) TLR6 mRNA was increased significantly after S.*aureus* stimulation ($P<0.001$); C) there was no difference in TLR6 mRNA in the control and treated macrophages.

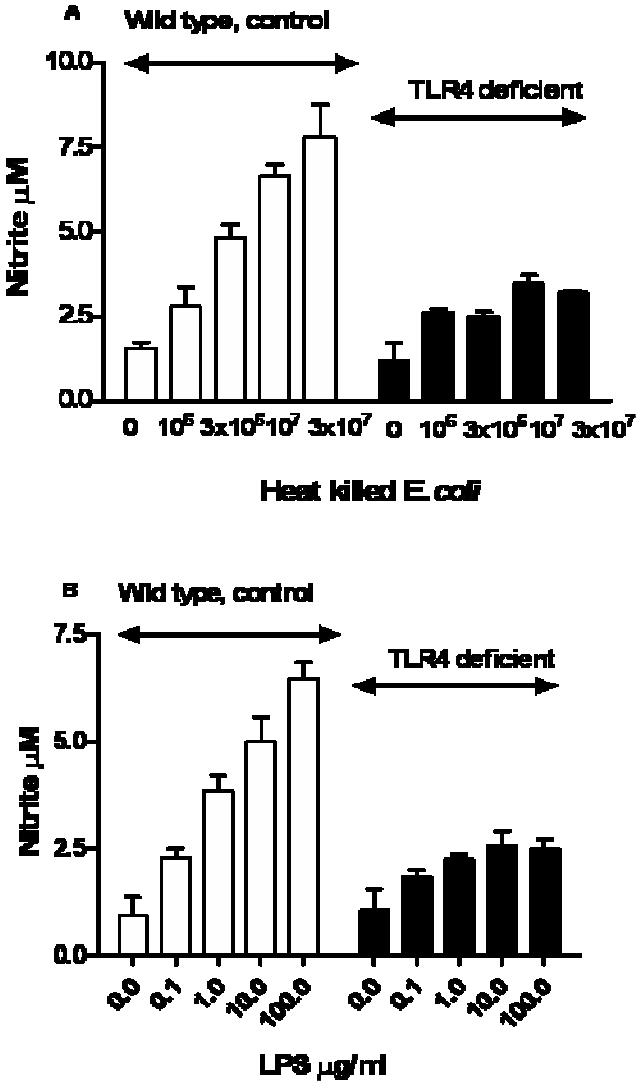

Fig. 7 Bone marrow TLR4$^{-/-}$ derived macrophages were less responsive to E.*coli* A) or LPS B) in terms of NO production.

2.3.2 Production of NO in vascular smooth muscle cells

iNOS is overexpressed in endothelial and vascular smooth muscle cells during an inflamed *status* like septic shock (5). We treated cultured vascular smooth muscle cells with S.*aureus* (10^5-10^8CFU/ml) and E.*coli* (10^5-10^8CFU/ml) for 24 hours.

In contrast to the macrophages, the Gram negative and Gram positive bacteria were able to induce NO release in a concentration dependent manner (Fig. 8A, B) on vascular smooth muscle cells. Furthermore, the analysis of western blotting revealed, as expected, the increase in NOSII protein expression (Fig.9) either after S.*aureus* (10^8CFU/ml) or after E.*coli* (10^8CFU/ml) stimulation.

Then, we analysed the amount of mRNA for TLRs in the murine aorta, cut in pieces of 5mm long and incubated with bacteria at 3 hours.

TLR1 and TLR6, responsible of S.*aureus* recognition, were significantly higher expressed than TLR2 and TLR4 (Fig.10) in the control aorta. TLR2 and TLR4 were relatively increased ($P<0.0001$, $P=0.09$, respectively) after the stimulation with S.*aureus* (10^8CFU/ml) and E.*coli* (10^8CFU/ml), respectively (Fig.11 A, B). In the same way, TLR1 and TLR6 mRNA were both increased after the bacteria treatment (Fig. C, D)

2.3.3 Comparison: macrophages *versus* vascular smooth muscle cells

S.*aureus*, did not induce the release of NO from macrophages, whilst E.*coli* or LPS did increase the production of NO through TLR4.

On the other side, vascular smooth muscle cells were sensitive to S.*aureus* as well as E.*coli* treatment, leading to NO release and iNOS protein expression.

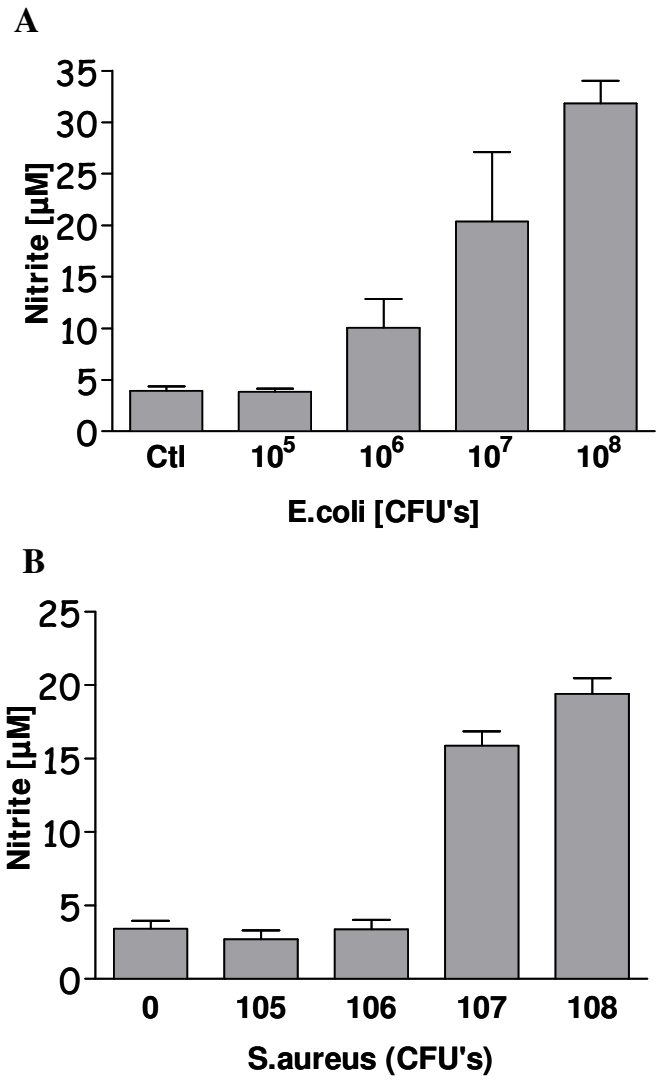

Fig.8 Rat aortic vascular smooth muscle cells released NO after A) E.*coli* and B) S.*aureus* stimulation.

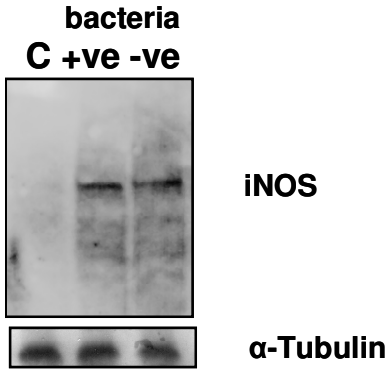

Fig.9 Either S.*aureus* (+ve) or E.*coli* (-ve) were able to induce iNOS expression on vascular smooth muscle cells at 24 hours.

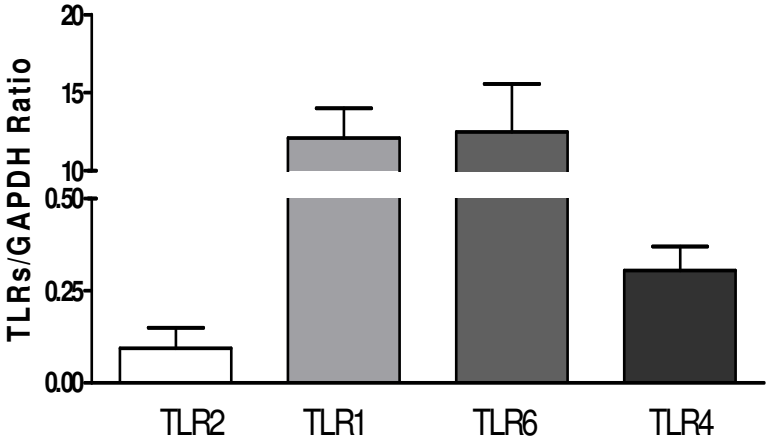

Fig. 10 Expression of TLRs in the murine aorta.

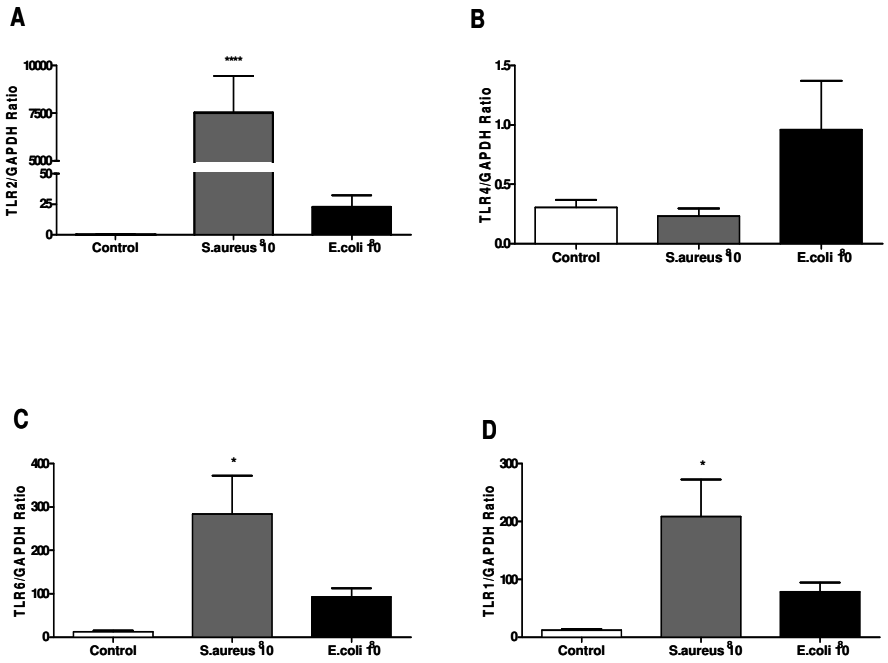

Fig. 11 A) TLR2 was highly expressed after S.*aureus* stimulation; B) TLR4 was not increased significantly after E.*coli* stimulation; C) TLR6 and D) TLR1 were significantly ($P<0.05$, $P<0.05$, respectively) increased after S.*aureus* treatment on the murine aorta.

2.4 Discussion

The expression of iNOS and subsequent "high-output" NO production underlies the systemic hypotension, inadequate tissue perfusion and organ failure associated with septic shock.

Macrophages are a major component of the mononuclear phagocyte system that consists of closely related cells of bone marrow origin, including blood monocytes, and tissue macrophages. From the blood, monocytes migrate into various tissues and transform into macrophages. In inflammation, macrophages have three major functions: antigen presentation, phagocytosis, and immunomodulation through production of various cytokines and growth factors. Therefore, macrophages play a critical role in the initiation, maintenance, and resolution of inflammation.

The infection of bacteria into the organism and the subsequent bacteria blood circulation can lead to macrophages activation and in the meanwhile the activation of stromal cells, like vascular smooth muscle cells, that have been recognised as able to 'sense' pathogens (13).

In this study we evaluated firstly the capability of either macrophages or vascular smooth muscle cells to produce NO in response to bacterial challenge, and secondly the difference between the two systems.

Macrophages were able to produce NO after E.*coli* treatment but not after S.*aureus* challenge. The lack of NO production after the Gram positive treatment was not due to a less responsiveness to that bacterium. In fact, macrophages produced the same amount of TNFα after S.*aureus* and E.*coli* administration.

TNFα and iNOS are key genes in innate immunity, which are thought to be regulated by separate arms of the MyD88-TRIF adapter protein pathway (14). As demonstrated by the bone marrow derived $TLR4^{-/-}$ macrophages, TLR4 was

implicated in the production of NO after E.*coli* and LPS challenge. In a recent study, it was showed that TNF release following TLR stimulation was achieved by NF-kB activation, whereas induction of iNOS was affected by NF-kB-induced IFN-β stimulation of IRF-3 (14). So, it was thought that while TNFα is induced following the activation of MyD88, iNOS requires TRIF activation. TRIF is a molecular adaptor protein which is not related to TLR2 activation. As S.*aureus* is sensed by TLR2, it could be possible that the lack of NO production at 24 hours was due to the lack of TRIF pathway activation on macrophages. Besides, it was shown that TNFα was able to act in an autocrine manner, leading to the induction of NO production (15). It is likely a later phase of TLR2-induced NO production. Accordingly, Clark et al (15) demonstrated that the stimulation at 48 and 72 hours of macrophages with S.*aureus* or PAMPs representative of the Gram positive bacteria can increase the amount of NO released. Besides, as TLR4 is coupled to either MyD88- or TRIF-dependent pathway (16), it could be reasonable that after E.*coli* administration, the NO production is first induced by TRIF involvement and later on by MyD88 after TNFα production (Fig 12).

Furthermore, the comparison between TLR2 and TLR4 gene expression highlighted that TLR2 was in a very low amount compared to TLR4 and that TLR2 mRNA increased after stimulation with the bacteria, in contrast to TLR4 mRNA expression which was not increased after the challenge, may be because of the already high level. These observations were in keeping with others (17) and let us to hypothesize that the activity of TLR4 is more prominent in macrophages, explaining the immediate reaction after E.*coli* challenge at 24 hours in these cells. Though, TLR2 expression was increased by S.*aureus* administration, further explanation for the involvement of MyD88 in TNFα release. Furthermore, these results are also in

keeping with studies which demonstrated a synergy between TLR2 and TLR4 agonists.

Vascular smooth muscle cells were able to produce NO either after E.*coli* or after S.*aureus* stimulation, result further confirmed by western blotting analysis for iNOS expression. TLR2 and TLR4 mRNA were increased after the respective bacteria treatment, leading to a concomitant response to the bacteria in the cardiovascular system. These data further confirms the role of NO in the vascular hyporesponsiveness to vasoconstrictors in *ex vivo* and *in vivo* vascular models of septic shock.

The principal aim of this study was to compare macrophages *versus* vascular smooth muscle cells in response to bacteria or PAMPs in terms of NO release.

A prominent difference in NO release between the two types of cells was observed after S.*aureus* challenge. Interestingly, the morbidity and mortality of septic patients has been found to be significantly higher in the presence of Gram positive bacteria, even though these organisms are conventionally considered to be weak pathogens. As demonstrated in this study, a possible explanation to this clinical event could be the non-immediate macrophages activation following the pathogen circulation. So, the pathogen, recognised by the vascular cells, can promote NO production, which in turn explicates a double effect. The immunocompetent cells release NO in order to kill the infectious agent, whereas the high output of NO from vascular cells induces vasodilatation which is deleterious in sepsis. The excessive production of NO by the vascular cells could still represent a sort of immuno-defence as they constitutively express TLRs, even though it becomes detrimental for the vascular reactivity and in particular for the blood pressure.

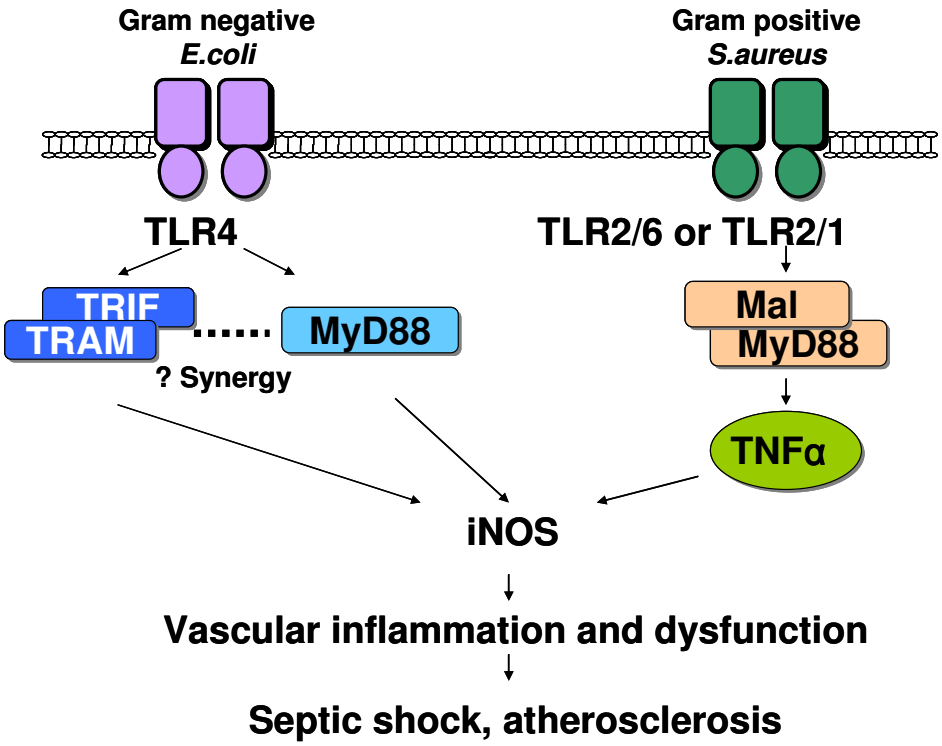

Fig. 12 Effect of Gram positive and Gram negative bacteria on iNOS induction.

References

1. Allary J, Annane D. Glucocorticoids and sepsis. *Minerva Anestesiol*. 2005; 71(12): 759-68

2. Schmidt HH, Pollock JS, Nakane M, Forstermann U, Murad F. Ca+2/calmodulin-regulated nitric oxide synthases. *Cell Calcium* 1992; 13(6-7): 427-34

3. Stuehr DJ. Mammalian nitric oxide synthases. *Biochim Biophys Acta* 1999; 1411, 217-230

4. Lincoln TM, Wu X, Sellak H, Dey N, Choi CS. Regulation of vascular smooth muscle cell phenotype by cyclic GMP and cyclic GMP-dependent protein kinase. *Front Biosc*. 2006; 11: 356-67

5. Evans T, Carpenter A, Kinermann H and Cohen J. *Circ Shock*.1993, 41: 77-81

6. MacMicking J, Xie QW, Nathan C. *Annu rev Immunol*. 1997; 15, 323-350

7. Singer M, Brealey D. Mitochondrial dysfunction in sepsis. *Biochem Soc Symp* 1999; 66: 149-66

8. Evgenov OV, Liaudet L. Role of nitrosative stress and activation of poly(ADP-ribose) polymerase-1 in cardiovascular failure associated with septic and haemorrhagic shock. *Curr Vasc Pharmacol.* 2005; 3(3): 293-9

9. Avontuur JA, Tutein Nolthenius RP. Prolonged inhibition of nitric oxide synthesis in severe septic shock: a clinical study. *Crit Care Med.* 1998; 26, 660-667

10. Ruetten H, Thiemermann C. Combination immunotherapy which neutralises the effects of TNF alpha and IL-1 beta attenuates the circulatory failure and multiple organ dysfunction caused by endotoxin in the rat. *J Physiol Pharmacol.* 1997; 48(4): 605-21

11. Lakhani SA, Bogue GW. Toll-like receptors signalling in sepsis. *Curr Opin Pediatr* 2003; 15(3): 278-82

12. Beutler B. Inferences, questions and possibilities in Toll-like receptor signalling. *Nature*. 2004; 430(6996): 257-63

13. Yang X, Coriolan D, Murthy V, Schultz K, Golenbock DT, Beasley D. Proinflammatory phenotype of vascular smooth muscle cells: role of efficient Toll-like receptor 4 signaling. *Am J Physiol Heart Circ Physiol.* 2005; 289(3): H1069-76.

14. Vogel SN, Fitzgerald KA and Fenton MJ. TLRs: differential adapter utilization by toll-like receptors mediates TLR-specific patterns of gene expression. *Mol Interv*. 2003; 3: 466-67

15. Paul-Clark MJ, McMaster S, Becher E, Sorrentino R, Anandarajah J, Fleet M, Srriskandan S, Mitchell JA. Differential effects of Gram-positive versus Gram-negative bacteria on NOSII and TNFalpha in macrophages: role of TLRs in synergy between the two. *Brit Journ Pharmacol.* 2006; 148, 1067-1075

16. Fitzegerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, Monks B, Pitha PM, Golenbock DT. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med.* 2003; 198(9): 1450

17. Moller AS, Ovstebo R, Haug KB, Joo GB, Westvik AB, Kierulf. Chemokine production and pattern recognition receptor (PRR) expression in whole blood stimulated with pathogen-associated molecular patterns (PAMPs). *Cytokine*. 2005, 32: 304-315

Chapter 3

Effect of hydrogen peroxide on human monocytes THP-1 cells

3.1 Introduction

Sepsis is a complex pathophysiological state and is still associated with a high degree of mortality. Gram negative and, increasingly, Gram positive bacteria are important causative agents (1). Infection results initially in the stimulation of the innate, non specific, immune response mediated mainly via circulating and tissue inflammatory cells such as monocytes/macrophages and neutrophils. These cells normally exist in a non-activated state but are rapidly activated in response to bacteria, their products or inflammatory mediators such as cytokines and reactive oxygen species (ROS) (2).

As most infections occur primarily in the tissue and not in the blood stream, extravasation of leukocytes is essential to recruit inflammatory cells and invading pathogens into contact. This requires both a chemotactic gradient and coordinated up-regulation of endothelial and inflammatory cell adhesion molecule expression. Leukocytes have a short life span at the inflammation site. Neutrophils rapidly undergo apoptosis to be cleared by inflammatory macrophages, which themselves emigrate from the inflamed site during the 'resolution phase'. Thus, a successful inflammatory event requires not only appropriate activation of cells and mediators with subsequent phagocytosis and removal of the exciting stimulus, but also a consequent elimination of the inflammatory cells and debris to allow tissues to reform a normal architecture and function (2).

Cytokines are presumably present to modulate cellular response and metabolism on a local or paracrine level. With few exceptions, like pre-pro-IL-1, cytokines have to be synthesized *de novo* in response to a specific external stimulus and do not exist in a dormant state inside the cell (2). However, once the cytokines are synthesized and secreted, they rapidly gain access to the blood stream. Septic patients displayed

high blood levels of TNF-α and IL-6, pro-inflammatory cytokines (3). Importantly, both pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and anti-inflammatory cytokines such as IL-10 and the soluble TNF-α receptor I and II coexist in the blood of patients with established sepsis (3).

The activation of various immune competent cells and non-immune competent cells, like epithelial or endothelial cells, and their consequent production of pro- and anti-inflammatory mediators, leads to the ROS release and other radical species with consequent oxidative stress. Several studies confirm severe oxidative stress in patients with systemic inflammatory response syndrome, like septic shock, as demonstrated by reduced values of plasma total radical-trapping antioxidant parameter and its components, such as uric acid, protein SH groups, unconjugated bilirubin, vitamin C, vitamin E and plasma unidentified antioxidants (4).

3.1.1 Role of Free Oxygen Radicals in the physiological control of cell function

The free oxygen radicals, also named ROS, are several and are classified as chemical species which can react with proteic, lipid and nucleotidic cell components either under physiological or pathological conditions (5).

ROS are superoxide anion $O_2^{-\cdot}$, hydrogen peroxide H_2O_2, hydroxyl radical $^{\cdot}OH$. Other reactive radical species, like peroxynitrite $ONOO^-$, frequent into the cell environment, are the reactive nitrogen species (RNS), which are formed by the reaction between nitric oxide (NO) and ROS.

The triplet-state molecular oxygen $^3O_2$, formed in an enzymatic way, such as via NAD(P)H oxidases or in a non-enzymatic way, is transformed into superoxide anion $O_2^{-\cdot}$. The enzyme superoxide dismutase (SOD) can convert the $O_2^{-\cdot}$ into H_2O_2, that can further, enzymacally or not, be transformed into the hydroxyl radical $^{\cdot}OH$.

Most of the cellular regulatory effects are not directly mediated by the superoxide but rather by its reactive oxygen species (ROS).

Free radicals and their derivatives exist in living tissues at low concentrations that are contained by the balance between the rates of radical production and their corresponding rates of clearance. The relative high intracellular concentrations of glutathione and other antioxidative compounds provide a strong basal scavenging capacity (5). Redox regulation can be defined as the modulation of protein activity by oxidation and reduction and has been recognized as one of the most important physiological mechanisms for controlling cellular activities. Under physiological conditions, an augmentation of ROS is a sensor of proteolytic degradation, oxygen homeostasis, and calcium changing concentrations, transcription factors activation, cell cycle regulation and so on. Thus, most of the redox-responsive regulatory mechanisms in mammalian cells serve to protect the cells against oxidative stress and to re-establish redox homeostasis (6).

3.1.2 Role of ROS in sepsis

Sepsis can activate various cells, such as macrophages, neutrophils, endothelial and epithelial cells, resulting in the release of a number of mediators, including cytokines, chemokines, leukotriens and proteases. This sequence of events leads to immune cells activation with the release of ROS. These inflammatory mediators are important to combat the pathogen but at the same time an overproduction of ROS leads to the oxidative stress, typical of pathologies like sepsis.

There are several evidences in the literature to confirm the progressing oxidative stress during sepsis, as registered by the increased level of the glutathione system activity and xanthine oxidase activity (7).

A further complication is the interaction between ROS and NO, highly produced in septic shock, leading to the production of RNS (5). RNS include NO, peroxynitrite $ONOO^-$, nitrogen dioxide radical ($NO_2^{\cdot}$) and other oxides of nitrogen and products arising when NO reacts with $O_2^{-\cdot}$, $RO^{\cdot}$ and $RO_2^{\cdot}$. Under physiological conditions, NO reacts rapidly with ferrous iron, binding to enzymes like soluble guanilate cyclase and cytochrome c of the respiratory chain, implicating a critical alteration of cellular components during an inflamed *status*. Furthermore, NO stimulates H_2O_2 and $O_2^{-\cdot}$ production by the mitochondria, possibly by inhibiting the cytochrome c oxidase, thus increasing leakage of electrons from the respiratory chain (2). H_2O_2, in turn, participates in the up-regulation of iNOS expression via NF-kB activation (8). NO can also bind to the haem of the enzyme catalase, inhibiting H_2O_2 breakdown.

The interaction of ROS and RNS with biological targets can therefore lead to lipid peroxidation, DNA damage, apoptosis and then to tissue injury (5).

3.1.3 Role of TLRs in the septic shock

Toll-like receptors are achieved as functional system of the innate immunity, capable of recognising pathogen-associated molecular patterns (PAMPs). The innate immune response has evolved as the immediate host defence system in response to foreign structures and it also serves to prime the adaptive immune response (9).

Several studies have identified the involvement of TLRs in the pathology of sepsis either *in vitro* or *in vivo* animal models. Escherichia *coli* and its outer membrane endotoxin lipopolysaccharide (LPS) are sensed by TLR4 either in macrophages or monocytes in an *in vitro* or *in vivo* model (10). In the same way, Staphilococcus

aureus or its membrane endotoxin LTA can be recognised by TLR2 (9), which heterodimerizes with TLR1 and TLR6. TLR2/1 complex is involved into the typical vascular hyporeactivity of sepsis, observed in aortic mice rings previously cultured with the Gram positive bacteria or specific TLR2 ligands (11).

Furthermore, the administration of TLR ligands on cell lines, like murine or human macrophages or vascular cells, revealed an increased production of cytokines like IL-1 and TNFα, and chemokines like CXCL-8.

3.1.4 Aim of the study

Under septic conditions monocytes are recruited from the blood into the injured tissues. The aim of this study was to evaluate the role of human monocytic THP-1 cells on the production of CXCL-8 after bacteria challenge and oxidative stress. We particularly focused on the activity of hydrogen peroxide (H_2O_2), typical ROS produced during the septic shock, on the production of CXCL-8. We then tried to examine the role and implication of TLRs on the oxidative stress induced by bacteria challenge on human monocytes THP-1.

3.2 Materials and methods

3.2.1 Culture and preparation of bacteria.

Bacteria were prepared in the same way as described in the paragraph 1.2.1.

3.2.2 Cell culture.

THP-1 human monocytes were obtained from the European Collection of Cell Cultures (ECACC) and cultured in RPMI 1640 containing 10mM GlutaMAX™ and supplemented with 10% FCS, Penicillin/streptomycin 100U/ml, L-glutamine 2mM. THP-1 cells were plated out onto either 6 or 96 well plates at 1 x 10^6 per ml in RPMI 1640 (0% FCS content), and left to equilibrate for 24 h before stimulation.

HEK 293 (human embryonic kidney cells) were obtained from Invivogen and cultured in Dulbecco's modified eagles medium (DMEM) supplemented with 10% FBS, Normocin 50mg/ml, Blestocidin 10mg/ml and non-essential aminoacids (1% v/v). HEK 293 TLR2, HEK TLR2/1 and HEK 293TLR2/6 were transfected with the respective TLRs and compared to HEK Null as control. The cells were plated onto 96-well plates at 1 x 10^6 per ml and let equilibrate 24 h before stimulation. The effect of all the reagents on THP-1 metabolism was assessed, by measuring the mitochondrial-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Poole, UK) to formazan. This was performed following all treatments.

3.2.3 Measurement of cytokine production.

CXCL-8 release in cell free supernatant were determined by Enzyme-Linked Immunosorbent Assay (ELISA) using commercially available matched antibody pairs following a protocol furnished by the manufacturers (R & D systems, Oxford,

UK). CXCL-8 concentrations were measured at 450 nm with a reference filter at 550 nm and results expressed as pg/ml.

3.2.4 Western blotting

The THP-1 cells were collected in the presence of protease and phosphatase inhibitors. Nuclear extracts kit (Activ Motif, UK) was used for the preparative purification of nuclear proteins. In order to separate cytoplasmic proteins from nuclear extract, hypotonic buffer was added to the cells followed by detergent causing leakage of cytoplasmic proteins into the supernatant. The cytoplasmic fraction was collected after centrifugation. The nuclear proteins were lysed by a lysis buffer containing a cocktail of protease inhibitors. The nuclear protein fraction was collected after centrifugation and analysed with Bradford assay for measuring the amount of proteins. Samples (30µg of proteins) were separated by gel electrophoresis on 10% SDS-polyacrylamide gels; after transfer onto nitrocellulose membranes Nuclear factor-Erythroid 2-related factor (Nrf-2) was detected using polyclonal rabbit antibody (1:1000; H-300; Santa Cruz Biotechnology, CA, USA) and the signal amplified with a goat anti-rabbit IgG-horseradish peroxidase (1:2000, DakoCytomation, Cambridge, UK). Blots were visualised onto film using ECL reagents (Amersham Biotechnology, Oxford, UK). The blots were then probed with Lamin A (1:1000; Santa Cruz, UK) that was used to confirm equal protein loading between lanes.

3.2.5 Reverse Polymerase Transcriptase and Real-Time Chain Reaction.

Total RNA was isolated from THP-1 cells (1.5x106 cells/ml in a 6-well plate) using the RNeasy Mini Kit (QIAGEN, Crawley, UK), after stimulation for 3 hours. cDNA was generated by reverse transcription using random hexamers. The cDNA (2µg/reaction) was used as a template in the subsequent polymerase chain reaction

(PCR) analyses. Transcript levels were determined by real-time PCR (Rotor Gene 3000; Corbett Research, Sydney, Australia) using the Sybr Green PCR Master Mix Reagent Kit (Promega, Madison, WI). The sequences of PCR primers were:

TLR2: sense, 5'-CCTCCAATCAGGCTTCTCTG;

antisense, 5'-CTCCCCTTGCAGATACCAT;

TLR4: sense, 5'-TGGACAGTTTCCCACATTGA;

antisense, 5' AAGCATTCCCACCTTTGTTG;

TLR 1: sense, 5'-TTGGCTGTGACTGTTGACCTC;

antisense, 5'-TGGCACACCATCCTGAGATA;

TLR 6, sense, 5'-TTGGCTGTGACTGTGACCTC;

antisense, 5'-TGGCACACCATCCTGAGATA;

GAPDH: sense, 5'-CAGCCTCAAGATCATCAGCA;

antisense, 5'-TGTGGTCATGAGTCCTTCCA.

Primers were used at a concentration of 1 μM for real time. Cycling conditions for real-time PCR (a total of 60 cycles used) were as follows: step 1, 15 min at 95°C; step 2, 25s at 60°C, 25s at 72°C; step 3, 5min at 72°C; step 4, 5s at 65°C to 95°C. Data from the reaction were collected and analyzed by the complementary computer software (Corbett Research). Relative quantifications of gene expression were calculated using standard curves and were normalized to GAPDH.

3.2.6 Statistical analysis

Prism 4.0c (GraphPad, inc) was used for all statistical analyses. All values are expressed as mean ± SEM. Student's t test was performed to statistically analyse the data. Values of p less than or equal to 0.05 were considered to be significant.

3.3 Results

3.3.1 Effect of bacteria on THP-1 cells

THP-1 cells were treated with E.*coli* and S.*aureus*, Gram negative and positive bacteria, respectively, at the concentration of 10^7 and 10^8 CFU/ml. Under basal culture conditions, the monocytes released a low or undetectable amount of CXCL-8. The treatment with E.*coli* induced a concentration-dependent release of chemochine (550±104.8; 15305±610.2 pg/ml) (Fig.2A), whereas S.*aureus* induced a detectable amount of CXCL-8 at the highest concentration considered (10^8 CFU/ml; 9545.42±1540 pg/ml) (Fig.2B).

ROS are derived from hydrogen peroxide (H_2O_2) and are especially formed during an inflamed *status* like sepsis. Therefore, we treated THP-1 with increasing concentrations of H_2O_2 (0.01-10mM). The H_2O_2 10mM was a very high concentration so that low cell vitality was detected. Thus, we used 0.1mM concentration which did not modify cell viability.

The addition of H_2O_2 0.1mM did not release any detectable CXCL-8. Interestingly, the addition of E.*coli* to H_2O_2 significantly potentiated CXCL-8 release at 10^7 ($P=0.05$) and 10^8 CFU/ml ($P<0.0005$) (Fig.2A). In the same way, the co-administration of S.*aureus* with H_2O_2 revealed a significant increase of CXCL-8 at the highest concentration 10^8 CFU/ml ($P<0.05$), revealing a more than additive effect (Fig.2B).

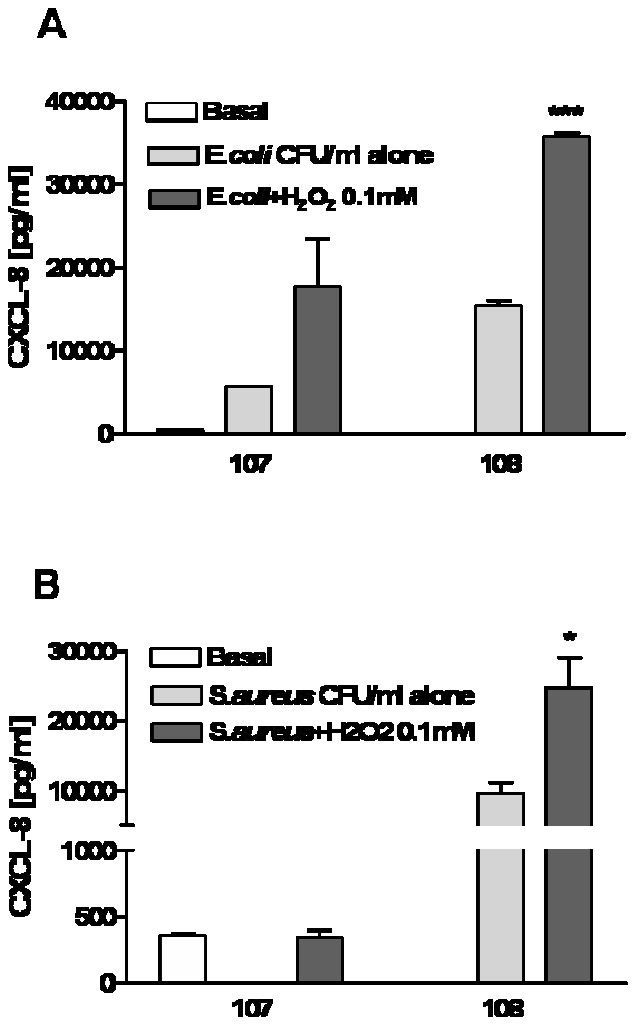

Fig. 2 A) E.*coli*+H_2O_2 increased significantly (10^7CFU/ml P=0.05; 10^8CFU/ml P<0.0005) the amount of CXCL-8 detected; B) S.*aureus*+ H_2O_2, in the same way, increased the detectable CXCL-8 (P<0.05) *versus* the basal (medium alone) on THP-1 cells.

3.3.2 Effect of PAMPs *plus* hydrogen peroxide on THP-1 cells

Lypopolisaccharide (LPS) is recognised by TLR4 and able to induce an inflammatory signalling pathway (9). We stimulated THP-1 cells with LPS 0.01 and 0.1 µg/ml for 24 hours. The amount of CXCL-8 registered was 1392.6±193.21 pg/ml at 0.01µg/ml and 5366.3±143.2 pg/ml at 0.1µg/ml. The co-addition of H_2O_2 with LPS significantly synergised the release of CXCL-8 at both concentrations tested ($P<0.0005$; $P<0.001$, respectively) (Fig.3A).

Pam3CSK4 and FSL-1 are specific TLR2/1 and TLR2/6 ligands. We administered these ligands in order to mimic the effect of S.*aureus* on TLR2 receptor and, especially on the heterodimers TLR2/1 and TLR2/6 as the bacteria can be 'sensed' by both complexes (9).

Pam3CSK4 (0.01-0.1µg/ml) and FSL-1 (0.1 and 1µg/ml) induced the release of CXCL-8, but, interestingly, the addition of H_2O_2 (0.1mM) potentiated the amount of chemokine detected either after Pam3CSK4 ($P<0.0005$ at 0.1µg/ml) or FSL-1 treatment ($P<0.001$ at 0.01µg/ml; $P<0.05$ at 0.1µg/ml) (Fig. 3B, C).

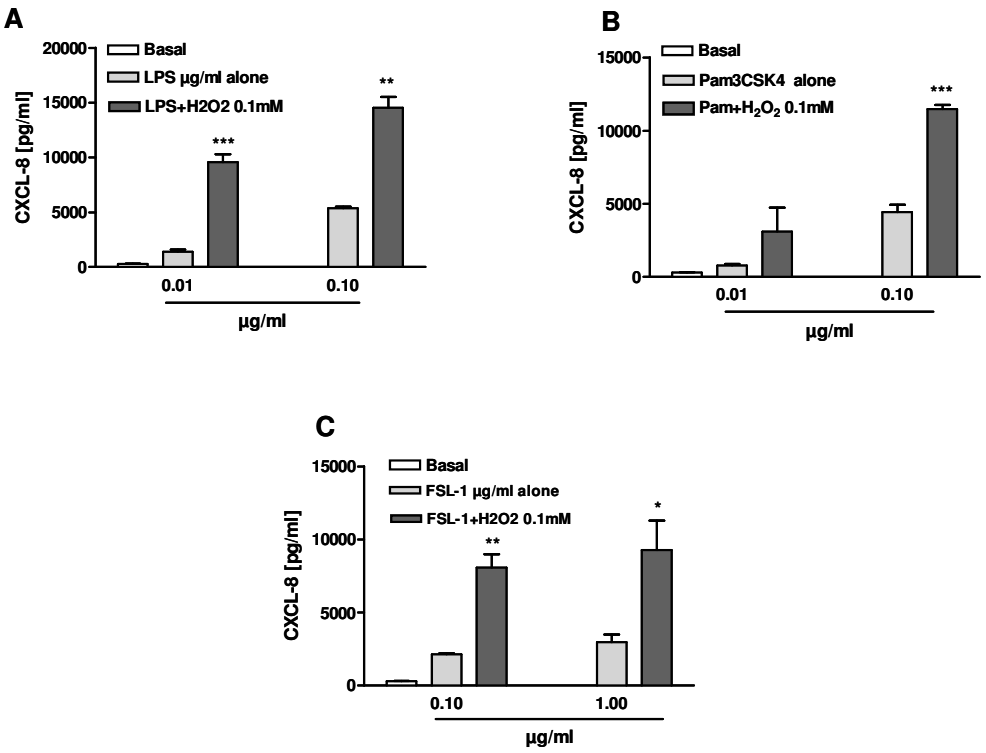

Fig.3 A) LPS+H_2O_2 significantly increased CXCL-8 detected at 0.01µg/ml ($P<0.0005$) and 0.1µg/ml ($P<0.001$); B) Pam3CSK4+H_2O_2 increased significantly CXCL-8 at 0.1µg/ml ($P<0.0005$), whilst C) FSL-1+H_2O_2 increased CXCL-8 in a significant manner at 0.1µg/ml ($P<0.001$) and at 1µg/ml ($P<0.05$).

3.3.3 Expression of TLRs on THP-1 cells

THP-1 cells are human monocytes cells, interested in the innate immunity. The expression of TLRs is constitutive as it is shown in the Fig. 4. TLR1 is highly expressed on THP-1 cells compared to the other TLRs.

We treated the cells with H_2O_2 to analyse which TLR mRNA was highly expressed. The stimulation with H_2O_2 revealed a significant increase in TLR4 ($P<0.05$), TLR2 ($P<0.005$) and TLR1 ($P<0.05$) mRNA, but not in TLR6 mRNA (Fig. 5A, B, C, D).

The co-administration of H_2O_2 to LPS (0.1µg/ml) revealed an increase in TLR4 mRNA (Fig. 6A), compared to the effect of the single compounds alone, even though it was not a significant additive effect. Meanwhile, the administration of H_2O_2 with FSL-1 (0.1µg/ml) revealed an additive effect on TLR2 but not TLR6 mRNA increase (Fig. 6B, D). In contrast, Pam3CSK4 (0.1µg/ml) *plus* H_2O_2 synergistically increased both TLR2 and TLR1 mRNA (Fig. 6C).

3.3.4 Effect of hydrogen peroxide and PAMPs on HEK 293 transfected cells

TLR2 heterodimerizes with TLR1 or TLR6. To further determine the role of TLR2 in the induction of CXCL-8, we cultured HEK 293 Null (non transfected), HEK TLR2, HEK TLR2/1 and HEK TLR2/6, transfected with the respective TLR. This kind of cells does not express any TLRs.

We stimulated the cells with H_2O_2 and interleukin-1β (IL-1, 1ng/ml). The H_2O_2 did not induce any significant increase of CXCL-8 compared to the basal conditions in all types of the HEK cells, as previously observed on THP-1 cell line. In contrast, HEK TLR2/1 cells showed a synergistic effect on CXCL-8 release after the H_2O_2 and IL-1 treatment (Fig. 7A, B, C and D).

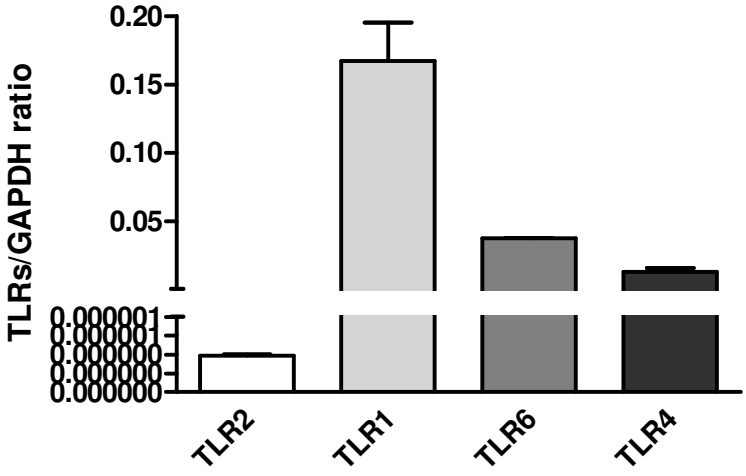

Fig. 4 Expression of mRNA for TLRs on THP-1 cells

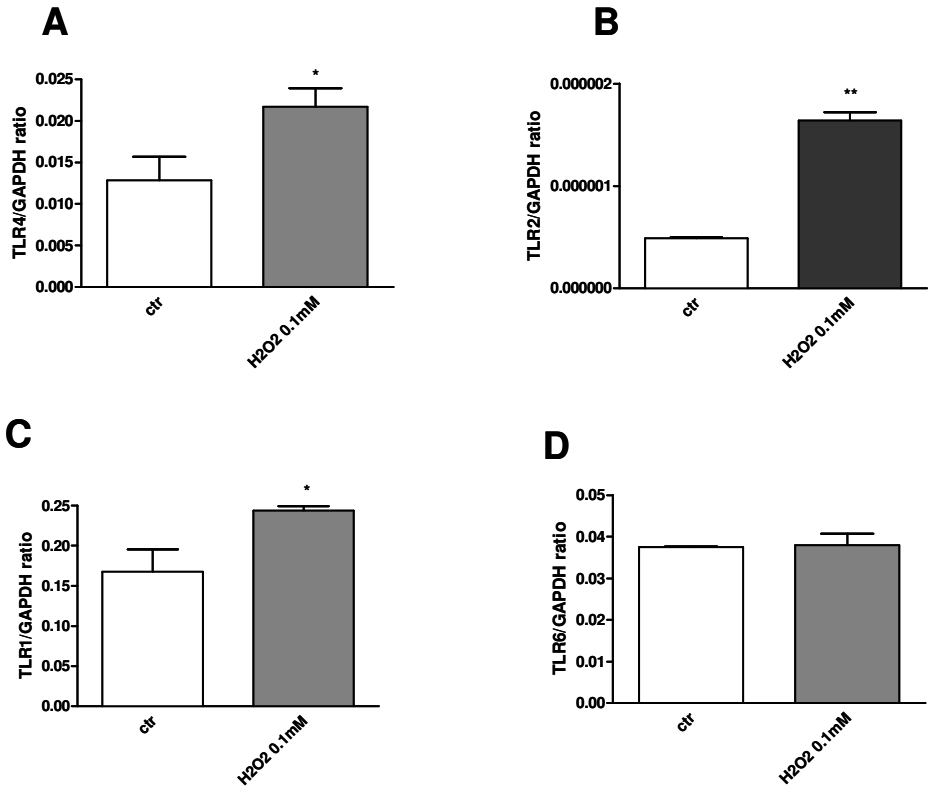

Fig. 5 H_2O_2 (0.1mM) increased significantly the amount of mRNA for A) TLR4 ($P<0.05$), B) TLR2 ($P<0.005$) and C) TLR1 ($P<0.05$), but not for D) TLR6.

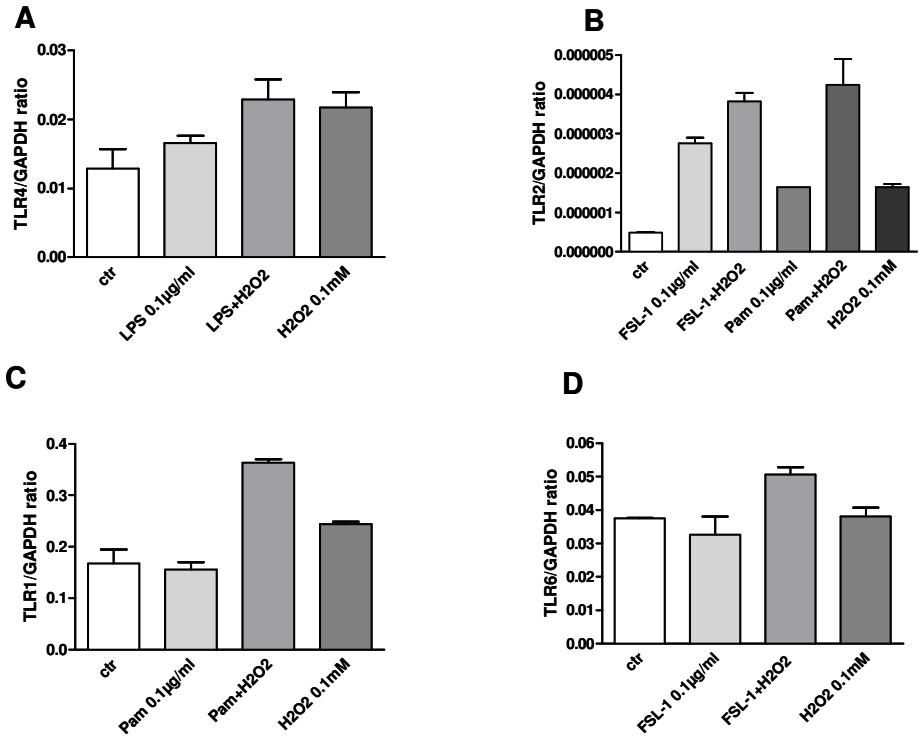

Fig. 6 The co-administration with PAMPs and H_2O_2 (0.1mM) increased mRNA for A) TLR4 and B) TLR2 in an additive manner after LPS (0.1µg/ml) and FSL-1 (0.1µg/ml) *plus* H_2O_2; whereas C) TLR1 mRNA was increased in a synergistic manner after Pam3CSK4 (0.1µg/ml) and H_2O_2 addition. D) TLR6 mRNA was not modified after FSL-1 and H_2O_2.

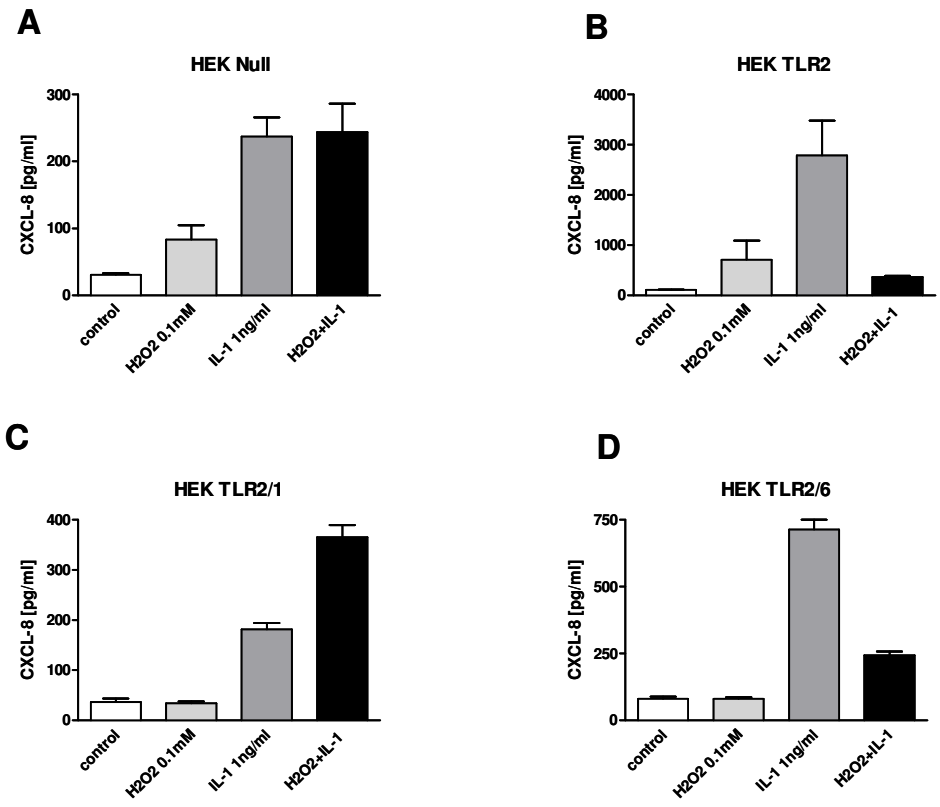

Fig. 7 HEK 293 A) Null and transfected with B) TLR2, C) TLR2/1 and D) TLR2/6 did not increase the amount of CXCL-8 after H_2O_2 treatment, but the addition of IL-1 increased the amount of CXCL-8 detected from HEK TLR2/1 in a synergistic manner.

3.3.5 Effect of PAMPs *plus* H_2O_2 on Nrf-2 expression.

Nuclear factor-Erythroid 2-related factor (Nrf-2) is an enzyme belonging to the family of the detoxifying enzymes, that are activated during the second phase after the insult from the oxidative stress. Under basal conditions, Nrf-2 is located in the cytoplasm, but in an oxidative stress *status* it translocates to the nucleus to induce the antioxidant response element (ARE).

Nrf-2 was low detected into the nucleus extraction proteins after H_2O_2 (0.1mM) treatment. N-acetylcysteine (1mM), a well known antioxidant, increased significantly ($P<0.05$) the amount of Nrf-2 compared to the control, especially it increased significantly the amount of Nrf-2 expressed into the nucleus after H_2O_2 treatment ($P<0.0001$) (Fig.8A). Interestingly, either LPS (0.1µg/ml) or Pam3CSK4 (0.1µg/ml) ($P<0.05$, $P<0.005$, respectively), but not FSL-1 (0.1µg/ml) significantly increased Nrf-2 into the nucleus compared to the control (Fig. 8B). Hence, the co-administration of H_2O_2 with LPS, FSL-1 and Pam3CSK4 increased the amount of Nrf-2 protein compared to the respective ligands alone ($P<0.001$; $P<0.01$; $P<0.05$, respectively) (Fig. 8B).

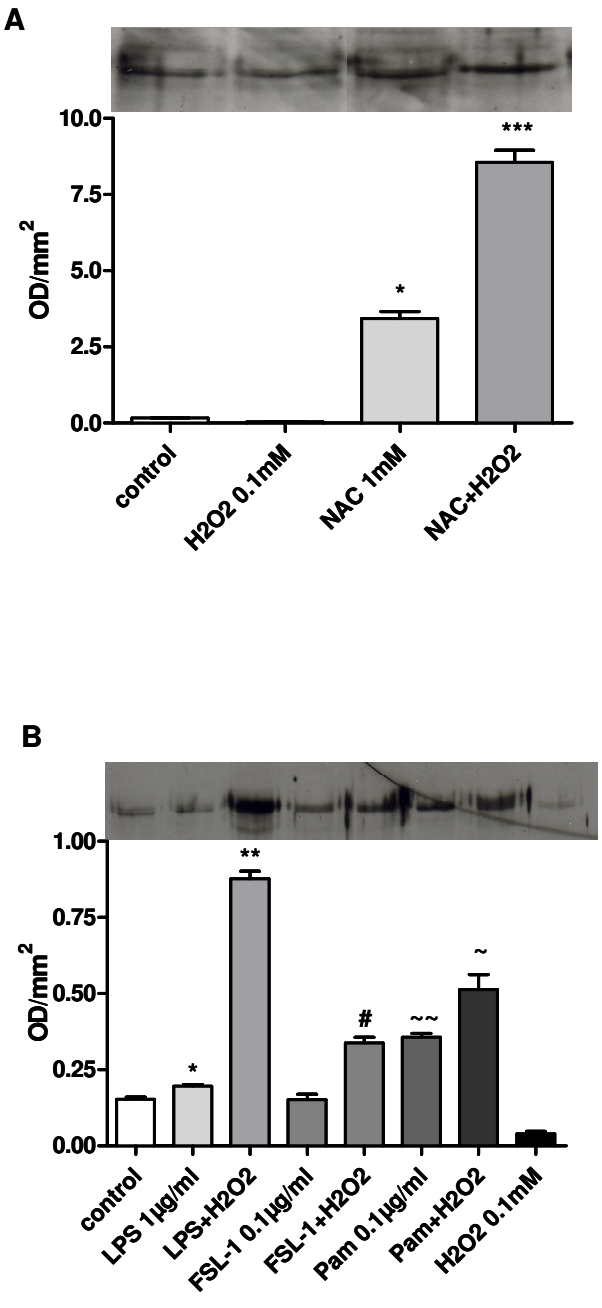

Fig. 8 A) N-acetylcysteine (NAC, 1mM) alone increased the expression of Nrf-2 into the nucleus (*P<0.05 *vs* control), as well as in combination with H_2O_2 (P<0.0001 vs NAC); B) LPS (0.1µg/ml) and Pam3CSK4 (0.1µg/ml) increased significantly Nrf-2 expression protein into the nucleus (*P<0.05, ˜˜P<0.005 *vs* control, respectively). LPS (**P<0.001 vs LPS), FSL-1 ($^{\#}$P<0.01 *vs* FSL-1) and Pam3CSK4 (˜P<0.05 *vs* Pam) *plus* H_2O_2 augmented Nrf-2 expression in a significant manner.

3.4 Discussion

Monocytes are cells that possess migratory, chemotactic, pinocytic and phagocytic activities, as well as receptors for IgG Fc-domains (FcγR) and iC3b complement (12). Under migration into tissues, they undergo further differentiation (at least one day) to become multifunctional tissue macrophages. Monocytes are generally, therefore, considered to be immature macrophages. However, it can be argued that monocytes represent the circulating macrophage population and should be considered fully functional for their location, changing phenotype in response to factors encountered in specific tissue after migration.

In this study we have been focused on the activity of monocytes to produce CXCL-8, the most potent chemoattractant for neutrophils. The administration of Gram negative, E.*coli*, or Gram positive bacteria, S.*aureus*, induced a concentration-dependent production of CXCL-8 but the addition of H_2O_2 revealed a synergistic effect. The administration of bacteria with H_2O_2 had the aim to mimic an oxidative stress condition (state), produced during septic shock. It is well-known that the amount of H_2O_2 produced in the organism is around 100μM under septic conditions and that it can interfere with the inflammatory response (5).

THP-1 cells were therefore stimulated with specific TLRs ligands and H_2O_2. It was confirmed the further involvement of TLRs in the pathogen-induced oxidative stress. LPS, TLR4 'sensed', *plus* H_2O_2 increased synergistically the amount of CXCL-8 produced as well as FSL-1, TLR2/6 ligand, and Pam3CSK4, TLR2/1 ligand. This data revealed that TLR4 and TLR2 are implicated in the exacerbation of the septic shock under oxidative stress conditions.

In order to further evaluate the above data, we analysed the effect of H_2O_2 on the expression of TLRs. TLR4, TLR2 and TLR1 mRNA were significantly increased after the administration of H_2O_2 alone, even though there was no detectable production of CXCL-8 after H_2O_2 cells stimulation. Accordingly, the addition of the PAMPs with H_2O_2 augmented the mRNA for TLR4, TLR2 and TLR1.

TLR4 was therefore responsible of the synergism previously observed after E.*coli* or LPS *plus* H_2O_2 treatment. According to our study, Powers et al (13) demonstrated that TLR4 was increasingly expressed on the surface of alveolar macrophages derived from a model of rat haemorrhagic shock. This effect was inhibited by the addition of the antioxidant N-acetylcysteine on RAW264.7 treated with H_2O_2 in vitro.

TLR2 is a receptor which heteredimerizes with TLR1 and TLR6. The complex TLR2/1 was responsible of the synergism observed after S.*aureus* and Pam3CSK4 *plus* H_2O_2 challenge, as further confirmed by the HEK transfected cells and real time PCR. Others demonstrated that TLR2 participated in the response of oxidative stress in cardiac myocytes via NF-kB and AP-1 activation, effect eliminated by the addition of TLR2 antibody (14).

In the second part of this study we evaluated the expression of Nrf-2 into the nucleus. Nrf-2 is a nuclear transcription factor which participates in the second phase of detoxification after induced oxidative stress. Under physiological conditions, nuclear levels of Nrf-2 are low, but they increase after oxidative stimuli, resulting in an enhanced transcriptional activation of its targets, which in turn confers protection against various environmental stresses (15). Nrf-2 activates the antioxidant response element (ARE), which induces the production of anti-oxidant

enzymes like glutathione, heme-oxygenase-1, which activity is to maintain the redox balance into the cell. This is the first study to evaluate that Nrf-2 was significantly expressed into the nucleus after LPS and Pam3CSK4 treatment. It was interesting to notice that Nrf-2 was even more expressed after LPS, FSL-1 and Pam3CSK4 *plus* H_2O_2 stimulation. Based on the anti-oxidant nature of Nrf-2, we could speculate that TLRs activation, in particular TLR4 and TLR2/1, had a double effect on monocytic cells. On one hand it promoted the recruitment of other anti-inflammatory cells, neutrophils by producing CXCL-8 via TLR4 and TLR2/1; on the other side they might activate a sort of down-regulation inducing a detoxifying phase inside the cells, resulting in a very balanced pattern. Interesting would be to discover at what point of the TLRs signalling pathway, Nrf-2 may interfere.

The role of Nrf-2 has been demonstrated crucial in a murine model of septic shock. Nrf-2 deficient mice were more susceptible to die after mild and lethal dose of LPS (16). Besides, $Nrf2^{-/-}$ mice expressed a greater amount of pro-inflammatory mediators, like IL-1 and IL-6 and TNFα, under septic conditions. In the same study it was demonstrated that Nrf-2 suppressed inflammation by inhibiting NF-kB activation through maintenance of redox *status* or maybe through the inhibition of IKK complex, responsible of NF-kB inactivity. NF-kB is a nuclear transcription factor induced after TLR4 and TLR2 activation. TLR4 signalling pathway, in contrast to TLR2, can be explicated via TRIF-dependent pathway, which is MyD88-independent pathway. It was demonstrated that Nrf-2 could also modify the activity of IRF-3-mediated gene transcription (15). IRF-3 is a downstream effector of TRIF-dependent pathway. The level at which Nrf-2 could act was also thought to be at the level of TRAF-associated NF-kB activator binding kinase 1 (TBK1).

Further studies are required to elucidate the probable mechanism of Nrf-2 and its cross-talk with the TLRs.

In conclusion, the activation of an Nrf-2 pathway could be useful to counterbalance the deleterious effect of TLRs on the synergistic production of CXCL-8. It could represent a sort of negative feedback for the extreme oxidative stress during the septic shock and, therefore, it may represent a future target for elaborating new therapies for sepsis.

References

1. Opal SM, Cohen J. Clinical gram-positive sepsis: does it fundamentally differ from gram-negative bacterial sepsis. *Crit. Care Med.* 1999, 27(8): 1608-16

2 Pinsky MR. Dysregulation of the immune response in severe sepsis. *Clin Invest* 2004; 328(4):220-9

3 Ghani RA, Zinudin S, Ctkong N, Rahman AF, Wafa SR, Mohamad M Manaf MR, Ismail R. Serum IL-6 and IL-1ra with sequential organ failure assessment scores in septic patients receiving high-volume haemofiltration and continous venus haemofiltration. *Nephrology* 2006; 11(5): 386-93

4 Marshall JC. The pathogenesis and molecular biology of sepsis. *Crit Care Resusc* 2006: 8(3): 227-9

5 Sakaguchi S, Furusawa S. Oxidative stress and septic shock: metabolic aspects of oxygen-derived free radicals generated in the liver during endotoxemia. *FEMS immunol Med Microbiol* 2006; 47: 167-177

6 Victor VM, Rocha M, De la Fluente M. Immune cells: free radicals and antioxidants in sepis. *Intern Immunolpharmacol* 2004: 327-347

7 Galley HF, Davies MJ, Webster NR. Xanthine oxidase and free radical generation in patients with sepsis syndrome. *Crit Care Med*. 1996: 24(10): 1649-53

8 Banan A, Zhang LJ, Shaikh M, Fields JZ, Farahadi A, Keskavarzian A. novel effect of NF-kappaB activation: carbonylation and nitration injury to cytoskeleton and disruption of monolayer barrier in intestinal epithelium. Am *J Physiol Cell Physiol*. 2004; 287(4): C1139-51

9 O'Neill LA. How Toll-like receptors signal: what we know and what we don't know. *Curr Opin Immunol* 2006; 18(1): 3-9

10 Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. Cutting edge: Toll-like receptor 4 (TLR4)-deficient are hyporponsive to lipolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 1999; 1; 162(7): 3749-52

11 Cartwright N, McMaster S, Sorrentino R, Paul-Clark M, Sriskandan S, ryffel B, Quesniauux V, Evans T, Mitchell JA. TLR2, TLR6, MyD88, TIRAP and TNF, but not TLR1 or TLR4 are required for the induction of vascular dysfunction by Gram positive S.*aureus. Shock* 2006

12 Stockinger H, Gadd SJ, Eher R, Majdic O, Schreiber W, Kasinrerk W, Strass B, Schnabl E, Knapp W. Molecular characterization and functional analysis of the leukocyte surface protein CD31. *J Immunol* 1990; 145(11): 3889-97

13 Powers KA, Szaszi K, Khadaroo RG, Tawadros PS, Marshall JC, Kapus A, Rotstein OD. Oxidative stress generated by hemorrhagic shock recruits Toll-like receptor 4 to the plasma membrane in macrophages. *J Exp Med*. 2006, 7; 203(8): 1951-61

14 Frantz S, Kelly RA, Bourchier T. Role of TLR-2 in the activation of nuclear factor kappa B by oxidative stress in cardiac myocytes. 2001, 16; 276(7): 5197-203

15 Kolls J. Oxidative stress in sepsis: a redox redux. *J Clin Invest* 2006; 116(4): 860-3

16 Thimmulappa RK, Lee H, Rangasamy T, Reddy SP, Yamamoto M, Kensler TW, Biswall S. Nrf-2 is a critical regulator of the innate immune response and survival during experimental sepsis. *J Clin Invest* 2006; 116(4): 984-95

Chapter 4

Human recombinant Erythropoietin reverts the vascular hyporesponsiveness of septic rats

4.1 Introduction

4.1.1 Sepsis

Sepsis is a devastating clinical condition that reverse its effect especially at the level of the cardiovascular system. It is the resultant of both local or systemic Gram-negative or positive bacteria infections and from translocation of the whole bacteria or endotoxin from the gut into the circulation (1, 2); it can convert to severe or septic shock and induce hypotension, multiple organ dysfunction, like acute lung injury, coagulation abnormalities, thrombocytopenia, altered mental status, renal, liver or cardiac failure.

The recognition of the endotoxins by the innate immune system leads to the activation and interaction of a number of effective cascades such as the complement, coagulation, bradykinin/kinin and hematopoietic systems, that can interact with each other and facilitate the release of a myriad of mediators in the acute phase response. These released mediators include eicosanoids, cytokines, chemokines, adhesion molecules, reactive free radicals, platelet-activating factor (PAF) and nitric oxide (NO).

Lypopolisaccharide (LPS) is the most common and experimentally studied cause of sepsis. Once in the circulation, LPS may bind to plasma components of the blood, such as high-density lipoproteins or LPS binding protein (LBP). The LBP-LPS complex interacts with CD14, a high affinity receptor for LPS expressed on monocytes/macrophages. The interaction with macrophafagic or blood soluble CD14 can stimulate endothelial cells, inducing the synthesis of adhesion-molecules which are then exposed to the membrane for recruiting leucocytes in the *focus* of the inflammation (2). The signal transduction, downstream LPS recognition, induces the activation of nuclear factor-kappa B (NF-kB). In physiological

conditions, NF-kB is normally associated and inhibited by the proteins Ikkα, β and γ, which form the inhibitory complex, IkB. An invasive stimulus, like LPS, induces tyrosine-kinase receptors activity which activates the IKK kinase, that phosphorilate IkBα, inducing this complex to dissociate. IkB is then recognized by the ubiquitin and degraded by the proteosome, whilst NF-kB migrates to the nucleus promoting the transcription of several genes, which express pro- and anti-inflammatory cytokines, inducible cyclo-oxygenase (COX-2), and inducible nitric oxide (iNOS) (Fig.1). This signalling pathway can be activated either by immunocompetent cells, like macrophages, or by stromal cells, like endothelial and epithelial cells.

Cytokines and chemokines are responsible of the recruitment and maturation of immune cells, leading to the activation of either the innate immune system or the adaptive immune system. Pro-inflammatory mediators, such as tumor necrosis factor α (TNF α), interleukin-1β (IL-1β), interferons (IFNs) and others, act either in an autocrine manner, auto-activating the immune cells which synthesized them, or in a paracrine-manner, inducing other cells to produce other enzymes implicated in the progression of the inflammation process.

However, the circulating cytokines are merely the 'tip of the iceberg'. Pro-inflammatory cytokines induce synthesis of phospholipase A2, COX-2, 5-lipoxygenase and acetyltransferase, which contribute to synthesis of eicosainoids, prostaglandins and leucotriens, and platelet-activating factor. These factors, acting through specific G-protein-coupled receptors promote inflammation, altering vasomotor tone and increasing blood flow and vascular permeability.

Furthermore, a variety of stimuli including cytokines, microbial components, immune complexes, and mechanical stress induce mRNA transcription and protein synthesis of iNOS. NO influences many aspects of the inflammatory cascade

ranging from its own production by immunocompetent cells to the recruitment of leukocytes. It can be synthesized by 3 NOS isoforms: NOS I, neuronal, and NOSIII, endothelial, which are constitutively expressed, and NOS II, inducible isoform transcripted via NFkB, previously activated by the inflammatory cytokines-induced signal transduction.

NOS converts L-arginine to L-cytrulline *plus* NO, which is a gas, that from the endothelial cells enters vascular smooth muscle cells, activating the soluble guanylate cyclase (sGC), which produces cyclic guanosin-monophosphate (cGMP), second messenger that promotes the activation of the cGMP-dependent protein kinase (PKG). In turn, PKG phosphorilates the light chain of the myosin in the smooth muscle cells, leading to the vasodilation. Physiologically, NO, the major vascular dilator produced by NOSIII, and vasoconstrictor mediators are strictly balanced, consequently modulating the vascular tone; but, an overproduction of NO in pathological conditions, like in sepsis, can damage the vascular endothelium and induce a massive vasodilation, hypotension and impaired tissue functionality with consequent multiple organ failure.

Further destructive NO-mediated effect is the consequent reaction with the reactive oxygen species (ROS). Physiologic generation of ROS, as described before, has been implicated in a variety of biological responses from transcriptional activation to cell proliferation. The overproduction of NO leads to the interaction with $O_2^{\bullet -}$ and H_2O_2, further generating reactive and toxic species like $ONOO^-$, extremely unstable and toxic for the cells. ROS have detrimental effects on endothelial function, vascular smooth muscle cell proliferation, and leukocyte adhesion. Oxidation of DNA and proteins may take place, along with membrane damage, because of lipid peroxidation, leading to alterations in membrane permeability,

modification of protein structure and functional changes (4). Oxidative mitochondrial membrane damage can also occur, resulting in membrane depolarization and the uncoupling of oxidative phosphorylation, with altered cellular respiration (5). This can ultimately lead to mitochondrial damage, with release of cytochrome c, activation of caspases and apoptosis (programmed cell death).

In terms of clinical conditions, all these biological effects are translated in massive vasodilation followed consequently by hypotension and multiple organ failure and death.

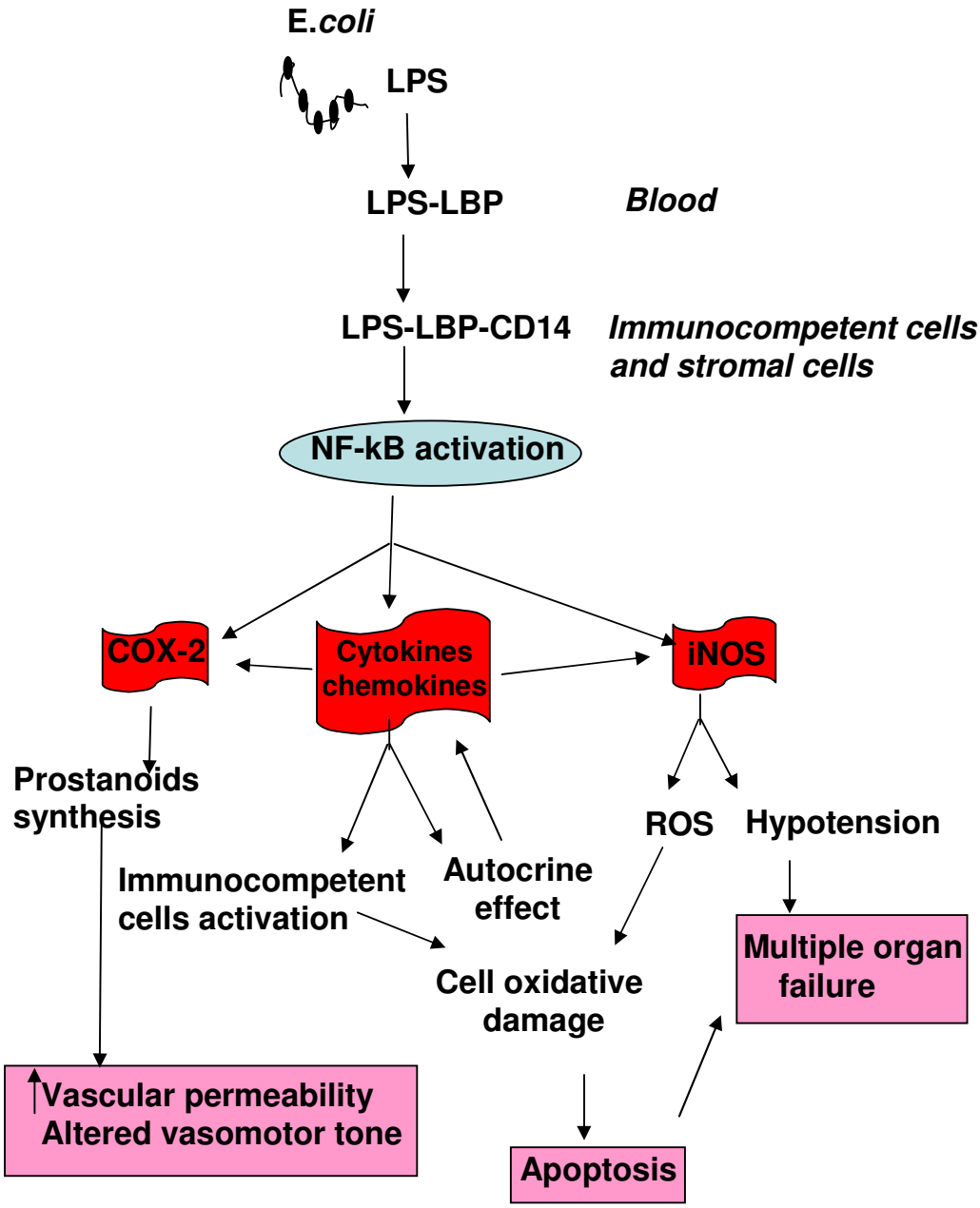

Fig.1 Effects of septic shock .

4.1.2 Erythropoietin

Erythropoietin (EPO) is a glycoprotein of 30.4 kDa, recently included in the great family of the cytokines. It is mainly produced by the hepatocytes during the fetal stage and after birth by peritubular fibroblast-like cell located in the cortex of the kidneys. EPO is the hematopoietic factor responsible of the production of red blood cells; in particular it promotes the proerythroblast survival and maturation. It is induced by tissue and blood hypoxia via the hypoxia-inducible factor (HIF), a transcription factor which exists as HIF-1α and HIF-1β. HIF-1α is located in the cytoplasm whilst HIF-1β is constantly present into the nucleus. Under hypoxic conditions, HIF-1α is enabled to enter the nucleus and heterodimerize with HIF-1β, active form for binding the hypoxia responsive elements (HRE) sequence, inducing the transcription of many genes like the EPO gene (6). Conversely, HIF-1 activation can be suppressed by haem ligands like carbon monoxide, nitric oxide and reactive oxygen intermediates (7). It has been reported that EPO mRNA expression stands an all-or-nothing fashion rather than a graded process in the renal cells (8).

Furthermore, there are other mechanisms that can regulate EPO gene transcription. In inflammatory diseases, GATA-2 and NFκ-B, nuclear transcription factors, are induced by the pro-inflammatory IL-1 and TNF-α, consequently contributing to the suppression of the EPO gene expression (Fig.2).

The mature EPO receptor (EPOR) is a glycoprotein, member of the cytokine class I receptor superfamily. The binding of the ligand to the receptor induces a conformational change and the activation of two molecules of Janus kinase 2 (JAK2), which are in contact with the cytoplasmic region of the receptor and, under the phosphorylated form, they induce the homodimerization of STAT5 (signal transducer and activator of transcription 5). Furthermore, the phosphorylation of the

residue of tyrosine of the EPO receptor induces the activation of either PI-3K/Akt or MAPK, kinase which is in turn activated by the phosphorylation of the domain SHC and consequent activation of Ras (9-10) (Fig. 3). The signalling cascade results in survival, proliferation and differentiation of erythrocitic progenitors. The EPO/EPOR complex is internalized and degraded. In addition, the action of EPO is terminated by hemopoietic cell phosphatase (HCP) which catalyzes the dephoshorylation of JAK2.

Recently, it has been demonstrated that recombinant human EPO (rhEPO) can exert its effects not only on the red blood cells, but also on cardiovascular and neuronal tissues (11-12). It seems that the growth factor explicates either cytoprotective or antiapoptotic effects (13-14).

Studies in literature revealed that EPORs are widely expressed in embryonic and adult tissues, including the central nervous system, gut, kidney, muscle (eg, smooth, skeletal, and heart), uterus, retina, pancreas, gonads, lung and cardiovascular system, including endothelial cells, smooth muscle cells and cardiomyocytes (15) Although the presence of EPORs on endothelium and vascular smooth muscle, the hormone had no direct vasoconstrictor effect on rabbit aorta and human renal artery, but it enhanced norepinephrine-induced contraction by increasing the synthesis of constrictor prostanoids and endothelin-1 (16). The probable source of these vasoconstrictor autacoids is the endothelium, since its removal attenuated the increase in contraction mediated by EPO. Furthermore, the EPO treatment caused an increase in $PGF_{2\alpha}$ and TXB_2 and a decrease in PGI_2 as well as an increase in the release of endothelin-1 in human umbilical vein endothelium cells. The incubation of bovine pulmonary arterial endothelial cells with rhEPO at 4 hours induced a rise of intracellular calcium concentrations accompanied by an increase in endothelin-1

and in prepro-endothelin-1 mRNA (17). On vascular smooth muscle cells EPO augmented either the mRNA or the functional expression of angiotensin receptor, affecting the vasomotor tone and the remodelling of vascular wall by enhancing cell proliferation (18). Furthermore, EPO also seemed to modulate cellular function by inhibiting apoptosis and inducing proliferation and differentiation (19). The observed *in vitro* cytoprotection seemed to be the basis of the protective effect of EPO showed in *in vivo* studies. Indeed, it was shown that EPO enhanced the survival of rats subjected to hypovolaemic hemorrhagic shock (20), to splanchnic artery occlusion and reperfusion (21) or myocardial ischemia and reperfusion (22).

4.1.3 Aim of the study

The aim of this study was to investigate whether rhEPO explicated a protective effect in the LPS induced-septic shock in the rat, by modulating the vascular dysfunction and explicating a cytoprotective effect. In particular we monitored the blood pressure values and *ex vivo* vascular and endothelial functions in aortic rings from LPS and LPS *plus* EPO treated rats.

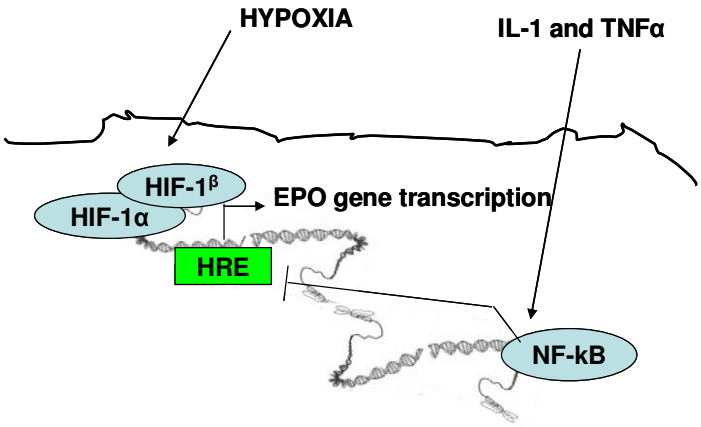

Fig. 2 Hypoxia induces EPO gene transcription, which in turn is blocked by NF-kB activation

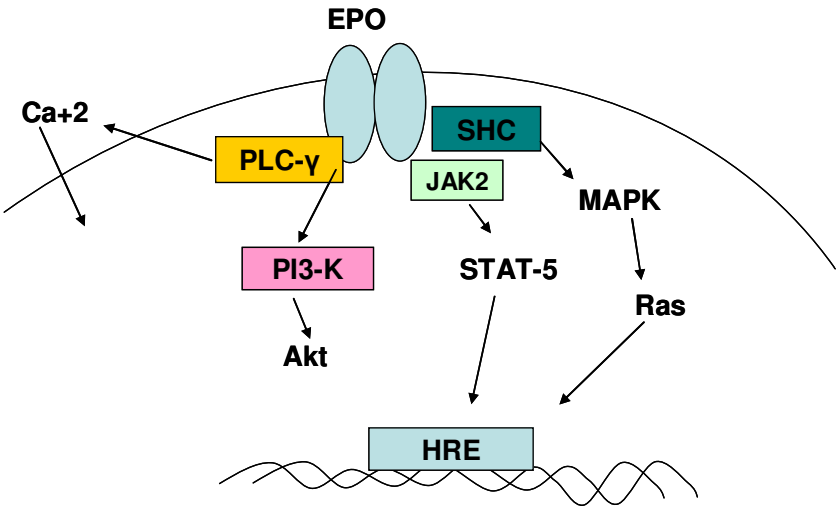

Fig. 3 Signalling pathway for erythropoietin receptor

4.2 Material and methods

Male Charles River rats (200-250g) were used for our *in vivo* and *ex vivo* experiments. Animals were housed under controlled conditions of light (12 hours light-dark cycle), humidity and temperature (21-24ºC) and had food and water *ad libitum*. A period of 7 days was allowed for acclimatization of rats before any experimental manipulation was undertaken.

Septic shock was induced by the injection of lipopolysaccharides from *Escherichia coli* (LPS, $8x10^6$ U/kg, i.v.; Sigma Aldrich).

Rats were randomly divided in four experimental groups: control (CTR), which received the vehicle (saline), control rhEPO (rhEPO Sham), which received the hormone only, LPS, endotoxemic animals and rhEPO *plus* LPS (rhEPO+LPS) which were treated with the hormone and LPS.

4.2.1 *In vivo* experiments

Human recombinant Erythropoietin (rhEPO, 300U/kg, i.v.; EPREX epoetinum alfa 6000 IU/0.6ml, Ortho Biotech) or vehicle (saline, NaCl 0.9%, i.v.) were administered 30 minutes before and 1 and 3 hours after LPS injection or saline. Treatments were performed in urethane (1g/kg, i.p.) anesthetized animals. After anaesthesia, trachea and right carotid artery were cannulated to facilitate the respiration and monitor mean arterial blood pressure (MAP), respectively. The carotid artery was connected to a pressure transducer (Bentley 800 Trantec, Basile, Comerio, Italy), by which MAP was continuously recorded using the Power Lab/800 System (AD/Instruments, Comerio, Italy). The left jugular vein was cannulated for the administration of drugs. Upon the completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 30 minutes, after which the above mentioned drugs were injected. MAP was monitored for all the

time of the treatment. At 4 hours after LPS injection, a dose–response effect by phenylephrine (PE 3, 10, and 30 μg/kg i.v.) was performed. The recover time between each injection was 15 min.

Arterial blood pressure is reported as mean arterial pressure (MAP) in mmHg.

4.2.2 Evaluation of red blood cells and haemoglobin

Peripheral blood was recovered from the four groups of animals from the rat tail. We evaluated the amount of red blood cells and haemoglobin by the Cell Dyne system. 40 μl of blood were aspirated by the machine and further diluted with an eluent solvent. Then, a small amount of diluted blood was aspirated by the machine and analysed in terms of red blood cells (RBC, k/μl)) and haemoglobin (Hgb, g/dl).

4.2.3 *Ex vivo* experiments

In another set of experiments rats treated with the same protocol were anesthetized by isoflurane (Abbott) for the *ex vivo* experiments. After 4 hours from LPS injection, animals were sacrificed by first exposure to isofluorane and then cervical dislocation. Thoracic aorta was taken out, cleaned and mounted in a 2.5 ml organ bath. After an equilibration period of 60 min and standardization of the tissue, the presence of the endothelium was evaluated by administering a dose-curve of acethylcoline (Ach 10nM-3μM) on PE pre-contracted aortic rings. In order to estimate the basal nitric oxide (NO) release, we administered NG-nitro-L-arginine methyl ester (100μM, L-NAME), irreversible nitric oxide synthase (NOS) inhibitor, on PE-induced contracted aortic rings.

To evaluate the vascular reactivity, we administered different vasoconstrictors like PE (1nM-3μM; Sigma Aldrich), angiotensin II (AGII, 0.1μM; Sigma Aldrich), endothelin (hET-1, 30nM; Tocris) and U-46619 (0.3μM; Sigma Aldrich), analogue stable of tromboxane.

4.2.4 Western blotting

For this set of experiments animals were treated and then sacrificed at 4 hours from LPS injection and thoracic aorta was taken out. Tissues were used immediately or stored at -80ºC.

The aortas were firstly mechanically disrupted by liquid nitrogen in the mortal and pestle, and then homogenated in a lysis buffer containing: HEPES 20 mM, MgCl2 1.5 mM, NaCl 0.4 mM, EDTA 1 mM, EGTA 1 mM, dithiothreitol 1 mM, phenyl methyl sulphonyl fluoride 0.5 mM, trypsin inhibitor 15 μg/ml, pepstatin 3 μg/ml, leupeptin 2 μg/ml, benzidamin 40 μM, nonidet P-40 1% and glycerol 20%.

The lysis buffer was left to react for 20 minutes on ice and then the protein concentration was estimated by the Bradford assay. Bradford reagent (Santa Cruz Biotechnologies) was diluted 1:4 with deionised water. A standard curve ranging from 0.05-0.5mg/ml bovine serum albumin (BSA, Sigma Aldrich) in phosphate buffered saline (PBS, 10mM) was prepared. Samples were diluted in PBS in order to fall within the range of the standard curve. 10μl of sample or standard was placed into a 96-well plate followed by 200μl of diluted Bradford reagent. The plate was read on a plate reader at λ=550 nm. All samples were then heated up to 95ºC for 5 min with a 2x gel loading buffer (50mM Tris, 10% w/v SDS, 10% v/v glycerol, 10% v/v 2-mercaptoethanol, 2mg/ml bromophenol blue) in a ratio 1:1, and centrifuged at 10000g for 10 min. Equal amounts of protein (30μg/ml) were run on a sodium dodecyl sulphate-polyacrylamide (SDS) gel electrophoresis gel (8% polyacrylamide) at 100 volts (V) for 15 min, to allow the samples to run through the stacking gel and concentrate into bands, and then at 150 V for 1 hour until the blue dye reached the bottom of the gel. It was also run a rainbow marker (Cell Signalling) as marker for the molecular weight. Then, the proteins were transferred

from the gel to a nitrocellulose membrane by using Transfer system for 1 hour at 100 V. Membranes were blocked for 40 min in PBS and 5% (w/v) non-fat milk and subsequently probed overnight at 4 °C with mouse monoclonal anti-iNOS (1:2500; Santa Cruz Biotechnologies) or mouse monoclonal anti-ICAM-1 (1:600; Santa Cruz Biotechnologies), mouse monoclonal anti-PARP (1:400; Santa Cruz Biotechnologies), anti-Bcl-2 (1:750; Santa Cruz Biotechnologies) and anti-Bcl-xl (1:750; Santa Cruz Biotechnologies) and monoclonal anti-mouse β-actin (1:2000; Sigma Aldrich). The unbound primary antibody was removed by 3 washing with PBS containing Tween 20 0.1%. Blots were then incubated with horseradish peroxidase conjugated goat anti-mouse immunoglobulin G (IgG; 1:2000) for 1 h at room temperature. Immunoreactive bands were visualized using electrochemiluminescence assay detection system (ECL, Amersham Biotechnologies) and exposed to Kodak X-Omat film. The protein bands of the target proteins were quantified by scanning densitometry using ImageJ program.

4.2.5 Statistical Analysis

The results are expressed as mean±s.e.m. and analysed using the two way ANOVA, followed by the Bonferroni post test, or the unpaired Student's t test. We considered significant P values less than 0.05.

4.3 Results

4.3.1 Effect of rhEPO on RBC and HgB

EPO is a growth factor, well-known to induce RBC maturation. We evaluated the amount of erythrocytes and haemoglobin (HgB) in the blood of rhEPO-treated rats. There was no difference in RBC and HgB amount after the treatment with rhEPO either in the EPO Sham or in the LPS+EPO animals (Fig. 4).

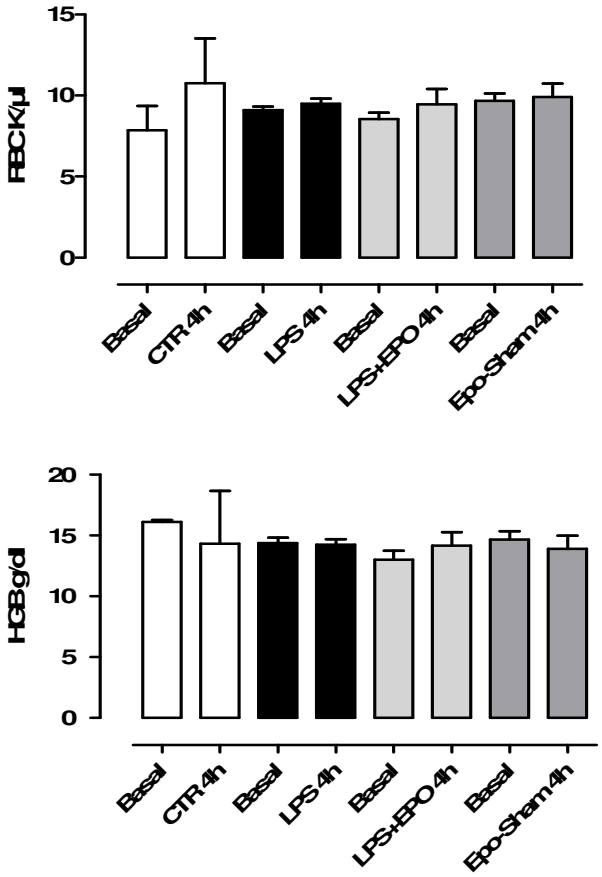

Fig.4 rhEPO did not modify RBC and HgB amount in our experimental condition.

4.3.2 Effect of rhEPO on rat blood pressure

LPS is the membrane lipopolysaccharide of *Escherichia Coli*, Gram negative bacteria, well-known to induce septic shock either in experimental models or in humans (13). We monitored the blood pressure for all the time of the experiment and evaluated the MAP values every 30 minutes since rhEPO or saline administration at the time -30. As expected, the injection of LPS induced a massive hypotension ($P<0.0005$) compared to the control rats. LPS was administered at the time point 0 and it induced a first immediate reduction followed by a more profound and constant decrease in the MAP. The immediate decrease in MAP was caused by the high viscosity of LPS solution, whilst the followed more profound hypotension was the real LPS systemic effect, observed by the low MAP values (79.5±4.2) *versus* the control (90.2±4.9). The administration of rhEPO recovered MAP values in a very significant manner ($P<0.0001$) compared to the endotoxemic rats (Fig. 5A). The effect of rhEPO alone did not affect the blood pressure.

After 4 hours LPS injection, we evaluated the vascular responsiveness by administering a dose-response curve of PE, adrenergic α1-agonist, in the jugular vein. As expected, the endotoxemic rats were significantly hyporesponsive to this vasoconstrictor compared to the control rat at the concentration of 3µg/ml ($P<0.005$) and 10µg/ml ($P<0.0005$). rhEPO reverted significantly the hyporeactivity of the LPS group of animals to the control values (3µg/ml $P<0.0001$; 10µg/ml and 30µg/ml $P<0.001$) (Fig.5B). rhEPO Sham rats reactivity to PE was comparable to the control values.

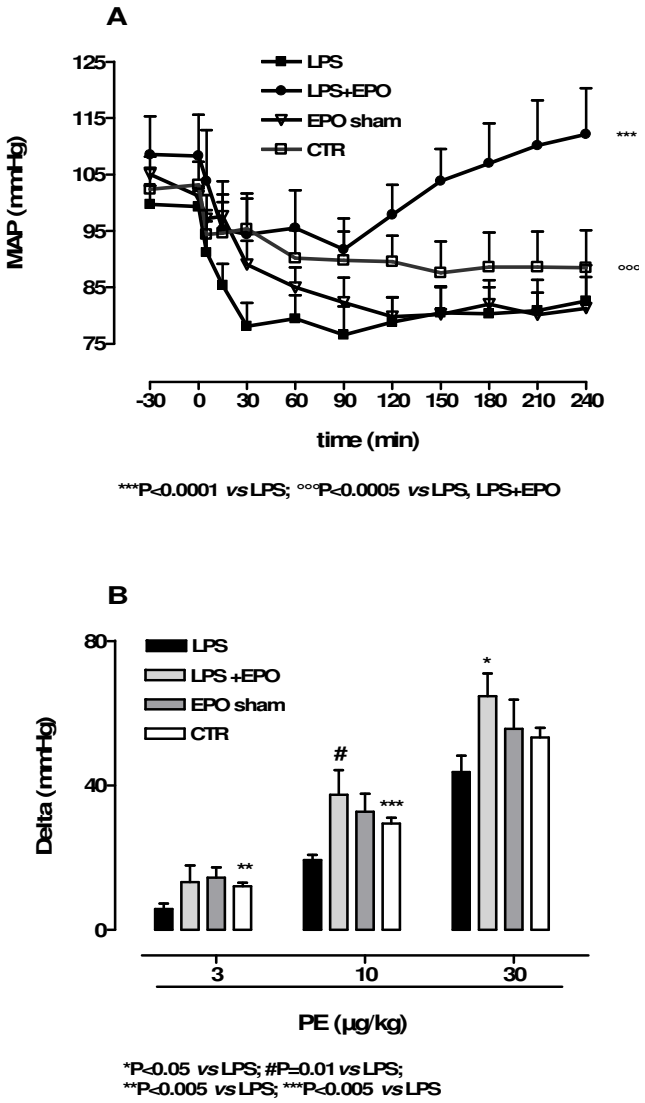

Fig.5 A) rhEPO significantly reverted the low values of MAP in septic rats (P<0.0001); B) rhEPO modified significantly the hyporesponsiveness to PE at 3µg/ml (P<0.005) and 10 µg/ml (P<0.0005) of septic animals.

4.3.3 The effect of rhEPO on the endothelial dysfunction in the rat aortic rings

The endothelium is exposed to the circulatory system, so it is more vulnerable to be damaged during the septic shock, by different mediators, like cytokines and ROS. We evaluated the aortic endothelial damage by administering Ach and L-NAME on a stable tone of PE. The concentration-response curve of Ach revealed the endothelial functionality whilst L-NAME administration showed the basal value of NO in the aorta after the different treatments. As expected, the endotoxemic aortic rings relaxed remarkably ($P<0.0001$) less than the control to Ach and, furthermore, revealed a greater amount ($P<0.01$) of NO than the control rings after L-NAME administration. Interestingly, rhEPO completely restored the endothelium functionality of rhEPO+LPS aortic rings to the control values (Fig.6A). The amount of the basal NO was reduced in a significant manner ($P<0.05$) in the LPS-treated aortic rings after rhEPO injection, almost similar to the control rings (Fig.6B), suggesting a probable lower iNOS activity.

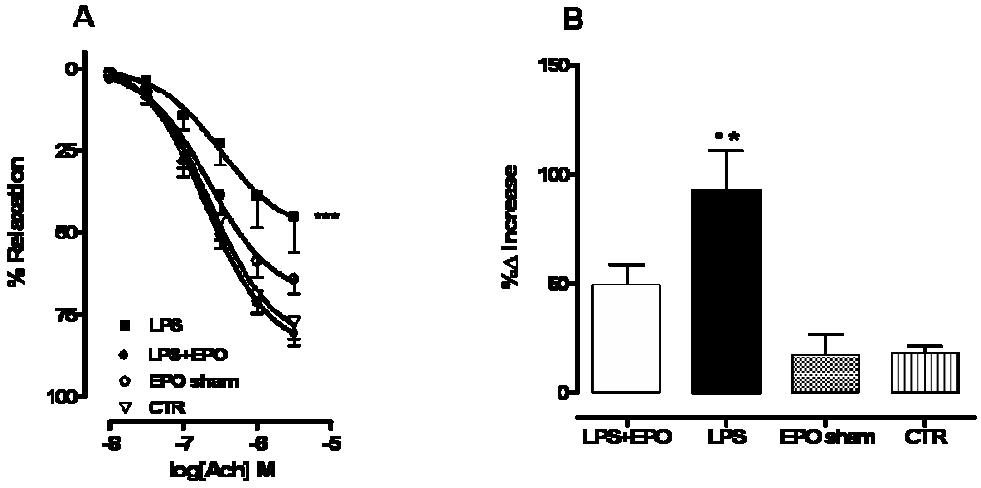

Fig. 6 A) rhEPO completely restored the endothelial impairment of septic aortic rings and B) decreased in a significant manner the high amount of NO.

4.3.4 Effect of rhEPO on aortic responsiveness to constrictor agents

Vascular hyporesponsiveness to vasoconstrictor agent is typical in the septic shock, due to the large amount of NO which damages the vascular smooth muscle cells. Recently, Akimoto et al. (22) demonstrated that rhEPO was able to increase calcium mobilization in rat vascular smooth muscle cells, leading to a synergistic effect on AGII, noradrenaline and ET-1 induced constriction. In order to evaluate the effect of rhEPO on the typical septic shock-induced vascular hyporeactivity, we administered several constrictor agents like PE, AG II, U46619 and hET1 on the aorta. Septic aortic rings were hyporesponsive to the concentration-response curve of PE compared to the control ($P<0.0001$) (Fig.7A). rhEPO treatment increased in a very significant manner ($P<0.0001$) the PE vascular reactivity of the septic aortic rings, comparable to the control rings. EPO Sham aortic rings were extremely reactive to the PE.

The same effect was observed with AGII administration. Endotoxemic aortic rings contracted less than the control rings ($P<0.005$) (Fig.7B). rhEPO treatment increased significantly ($P<0.05$) the responsiveness to AG II in the septic aorta, whilst EPO sham aortic rings contracted in the same way as the control rings.

Then, we administered hET1 (30nM) and as expected, endotoxemic aortic rings were hyporesponsive to ET1, compared to the control or EPO Sham ($P<0.005$). In contrast, LPS+EPO aortic rings showed a significant ($P<0.05$) increased contractility to hET1 than the respective endotoxemic aortic rings (Fig.8A).

Furthermore, rhEPO treatment was able to revert ($P<0.05$) the hyporesponsiveness of endotoxemic rats to U46619, leading to a contractility similar to the control or EPO Sham values (Fig.8B).

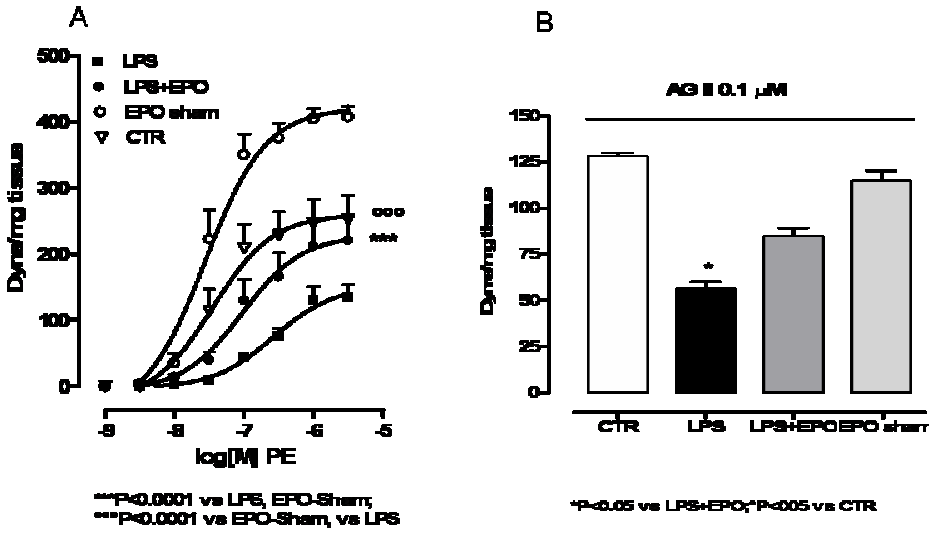

Fig.7 rhEPO increased the dose-concenration response to the A) PE and B) increased the reactivity to AG II (0.1μM) of septic aortic rings.

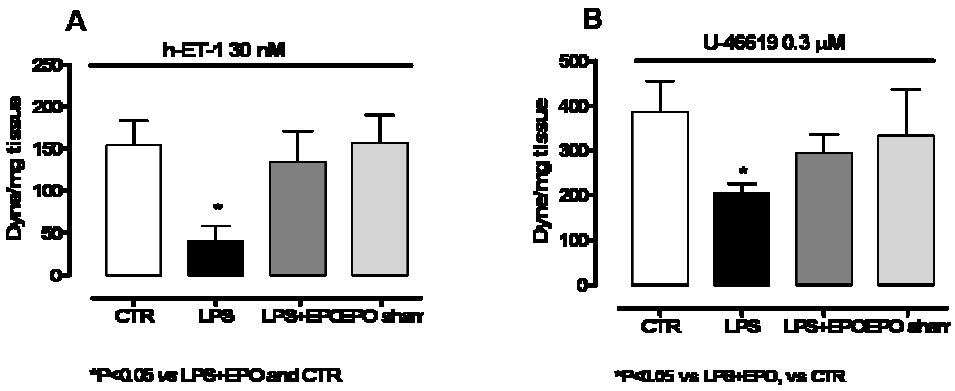

Fig.8 rhEPO increased the reactivity to A) h-ET-1 30nM and B) U46619 0.3μM of the septic aortic rings.

4.3.5 Effect of rhEPO on the aorta protein expressions

In order to understand the mechanism by which rhEPO was able to revert the endothelial damage we investigated on the iNOS expression. As it is shown in the figure 9A, iNOS, inducible isoform of NOS, was very highly expressed in the endotoxemic aorta compared to the control ($P<0.005$). rhEPO decreased significantly ($P<0.005$) iNOS expression in septic aorta at similar values as the control.

ICAM-1 is an adhesion molecule that facilitates the diapedesis of leucocytes through the endothelium. Its expression was highly expressed in endotoxemic aorta compared to the control. rhEPO treatment diminished in a significant manner ($P<0.05$) ICAM-1 expression (Fig 9B). Either the control or the EPO Sham aortic rings did not show great amount of this protein.

Poly(ADP-ribose)polymerase 1 (PARP-1) is an abundant nuclear chromatin-associated protein and its activation in severe sepsis has emerged as one of the central mechanisms of systemic inflammation, endothelial dysfunction, peripheral vascular failure, and reduction of cardiac contractility (23). rhEPO treatment decreased in a remarkable manner the expression of this enzyme ($P<0.05$) in the endotoxemic aorta, leading to the same values expressed in the control and EPO Sham aorta (Fig 9C).

Recently, it was shown that EPO can explicate cytoprotective effects but also antiapoptotic effects. We evaluated Bcl-2 and Bcl-xl expression as antiapoptotic markers. LPS aorta showed a very low amount of both Bcl-2 and Bcl-xl compared to the control ($P<0.01$). rhEPO increased the amount of these antiapoptotic proteins ($P<0.01$ and $P<0.001$) (Fig 10 A and B).

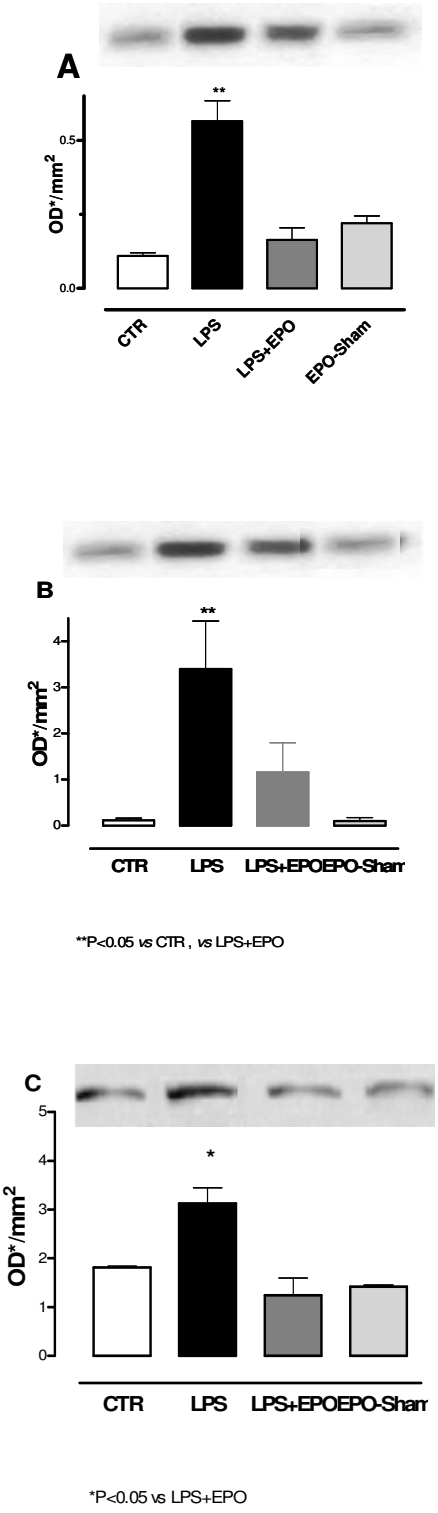

Fig. 9 rhEPO decreased A) iNOS, B) ICAM-1 and C) PARP expression in a significant manner in the septic aortic rings

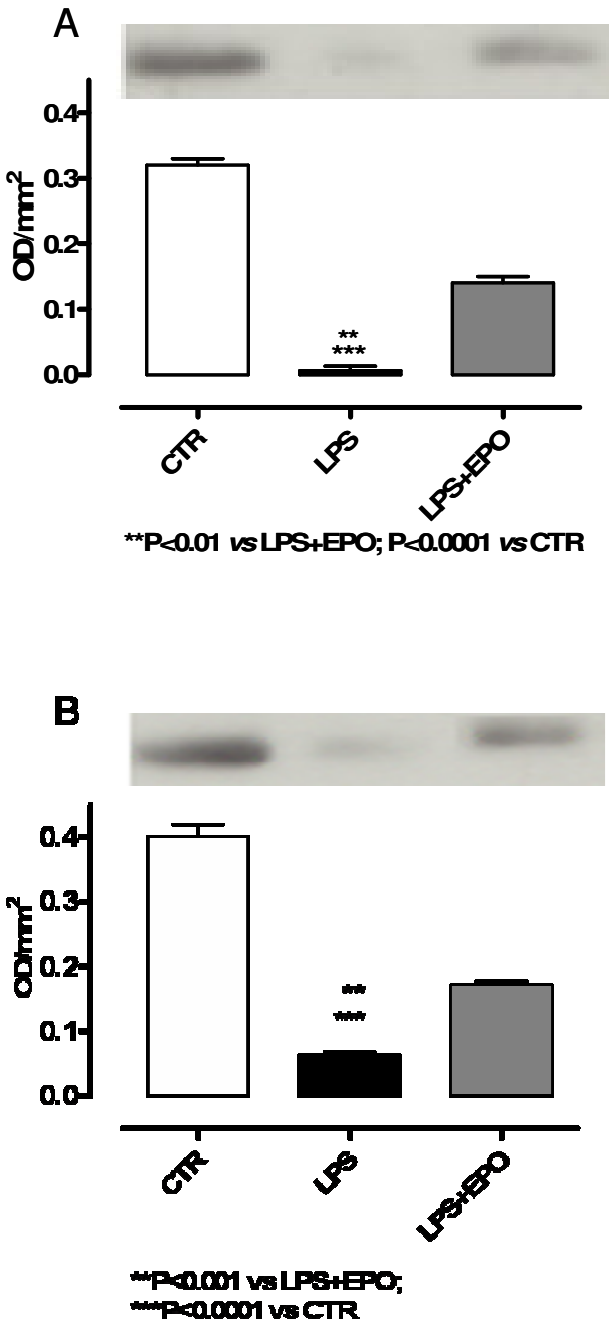

Fig. 10 rhEPO decreases the aortic levels of A) Bcl-2 and B) Bcl-xl in the septic rats

4.4 Discussion

Human recombinant erythropoietin is currently used in the standard therapy for correction of renal and non-renal anaemia. rhEPO can correct the anaemia derived from the renal failure but can also be administered in case of anaemia associated with cancer chemotherapy, autoimmune diseases, bone marrow transplantation and myelodysplastic syndromes (13). However, besides its hematopoietic effects it has recently been suggested that rhEPO has a beneficial role in the cardiovascular system, preventing damages from heart ischemia-reperfusion (22), haemorrhagic shock (20) and splanchnic artery occlusion shock (21). Our study revealed a beneficial effect of rhEPO in the LPS-induced septic shock in the rat. In our experimental conditions, the administration of rhEPO reverted either *in vivo* vascular hyporesponsiveness or *in vitro* vascular hyporeactivity to vasoconstrictor agents. The *in vivo* experiments showed the beneficial effect of rhEPO in reverting the fall in MAP registered in septic rats. Consistent to our results, Squadrito et al (21) demonstrated that the hormone was able to increase the MAP and restore the survival rate of rats injured by a model of splanchnic artery occlusion. In addition, our results revealed that rhEPO was also able to revert the typical septic hyporesponsiveness to the PE when injected via the jugular vein. Analogous result was observed *ex vivo*, remarking the capability of the cytokine to revert the hyporeactivity to the vasoconstrictor reagents PE, AG II, U46619 and hET-1. In LPS+EPO group we observed a vascular responsiveness similar to the control group suggesting the EPO capability of reverting the typical LPS-induced hyporeactivity.

One of the side effect of rhEPO used for the chronic anemia therapy is the increased blood pressure. We administered the hormone in an acute manner and it did not affect the physiological responsiveness to vasoconstrictor reagents as shown by EPO Sham group of animals either in the *in vivo* or in the *in vitro* experiments. Furthermore, in order to evaluate whether rhEPO could alter the basal and physiological vascular tone, we administered the hormone 20 minutes before PE on the aortic rat rings and noticed that it did not modify the vascular response to the vasoconstrictor.

Several could be the mechanisms to explain rhEPO capability to restablish the vascular reactivity in the septic shock, like the increased influx of calcium or the increased gene transcription. Recently, it has been shown that rhEPO stimulates a dose-dependent increase in the intracellular free calcium ($[Ca^{2+}]_i$) through a voltage-independent ion channel (23). It modulates Ca^{2+} influx through the transient receptor potential (TRP) protein family member TRPC2 (24). TRCP can activate different isoforms of phospholipase C (PLC) (24), resulting in inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) formation. Some TRPC are Ca^{2+} store release operated and can be activated by IP3. A common characteristic of many TRPC channels is that they are activated through pathways involving PLC. As EPO also activates PLCγ1 and PLCγ2 (25, 26), the activation of TRPC2 through a PLC-dependent pathway could be required for stimulation of Ca^{2+} influx, resulting in the increased vascular reactivity observed in the *in vivo* and *ex vivo* experiments.

Another possible mechanism could be the modulation of the DNA synthesis. Studies in literature and our data demonstrated that rhEPO can increase the response to AG II. Vascular smooth muscle cells cultured with rhEPO (6-8

units/ml) showed elevations (40-120%) in messenger RNAs of the renin-angiotensin system (renin, angiotensinogen, angiotensin receptor types 1 and 2) and increased levels of several messenger RNAs known to respond to angiotensin II (transforming growth factor-beta, insulin-like growth factor-II, epidermal growth factor, c-fos and platelet-derived growth factor) (27). The peptide Angiotensin II (Ang II) participates in the control of the systemic arterial pressure increasing smooth muscle tone and acting on the smooth muscle cell DNA synthesis. As described by Villanova et al., one of the target of AG II could be the induced expression of alpha(1)-adrenoceptor, especially the alpha(1D) subtype (28), hypothesis that could either explain the augmented reactivity to the AG II and PE of EPO+LPS rats or the increased reactivity to the PE in the EPO Sham group of animal.

Furthermore, Bode-Boger et al (16) demonstrated that rhEPO was able to induce the release of vasoconstrictor agents, like endothelin and tromboxane B2, from incubated HUVEC and rabbit artery. Consistently to this study, we observed that rhEPO was also able to increase the aortic responsiveness to hET-1 and U46619. This increased vascular reactivity could be due to either an augmented gene transcription for the two above constrictors, or to a synergistic effect of rhEPO in inducing an increased Ca+2 influx, which is well-known to participate to the vascular contraction pathway.

Another aspect of the rhEPO cardiovascular protection in our model of septic shock was the prevention of the endothelial dysfunction. As reported in Figure 6, septic rat aortic rings were characterized by a profound endothelial damage showed by the hyposponsiveness to the dose-curve of Ach and high amount

of NO levels. rhEPO reverted the endothelial damage and decreased the amount of basal NO by reducing the effect of L-NAME on the stable tone of PE in the endotoxemic rings. To further demonstrate the beneficial effect of rhEPO on the impaired endothelium, we considered two biological markers, iNOS and ICAM-1 protein expression. iNOS activation induces an overproduction of NO leading either to a massive vasodilation or to an intense ROS formation. Meanwhile, ICAM-1 expression promotes capillary permeability inducing to the diapedesis of many inflammatory cells, like the leucocytes, to the injured tissue. rhEPO decreased in a very significant manner the amount of iNOS and ICAM-1 expressed in the endotoxemic aortae. In the same way, Squadrito et al (21) demonstrated that rhEPO reduced plasma nitrite/nitrate, serum TNFα and iNOS activity in both aorta and peritoneal macrophages of rats after splanchnic artery occlusion shock.

Further interesting result was to evaluate the antiapoptotic effect of rhEPO in the septic shock. Indeed, rhEPO decreased the activity of PARP whilst increased the amounts of Bcl-2 and Bcl-xl in the aorta.

Poly(ADP-ribose) metabolism plays a role in a wide range of biological structures and processes, including DNA repair and maintenance of genomic stability, coordinating or participating in repair or apoptosis. This enzyme is activated as a consequence of the DNA damage, typical of the septic shock as many ROS are produced and can interfere with the DNA structure. Its activation induces to repair the DNA structure or to the apoptosis if the damage is not repairable. The reduction in PARP expression in EPO+LPS rats indicated that rhEPO induced a reduction in the LPS-induced cellular damage.

In addition, rhEPO increased the aortic levels of Bcl-2 and Bcl-xl, which are antiapoptotic proteins. The activation of these 2 proteins is a consequence of a strict balance between pro- and anti-apoptotic proteins and are usually complexed with BAX, a pro-apoptotic protein associated to the mitochondrial membrane. The dissociation from this inhibitory complex induces Bcl-2 and Bcl-xl to inhibit the initiated apoptotic process. It is already known that EPO can prevent the apoptosis of erythroid precursor cells via its own receptor (29). A recent study highlighted its antiapoptotic effect on vascular smooth muscle cells (VSMC) stimulated with IL-1β, known to be able to induce large amount of NO which in turn can stimulate the apoptotic process (30). Akimoto et al. demonstrated that rhEPO can activate PI3-kinase and induce Akt phosphorylation, namely Akt activation, through PI3-kinase-dependent pathway in VSMCs. PI3K/Akt pathway leads to the upregulation of Bcl-xl protein family and inhibition of apoptosis in Baf-3, hematopoietic cells (31), and furthermore, can induce the translocation of NF-kB into the nucleus, preventing the apoptosis in the injured hippocampal neurons (32). NF-kB is reported to be dually activated by JAK2 and Akt (33), increasing the production of the antiapoptotic Bcl-xl family and preserving the integrity of the DNA (34).

In conclusion our study revealed the beneficial and protective effect of rhEPO in the septic shock. Its protective effect is explained through several pathways downstream EPOR activation. Summarizing, the massive hyporeactivity to vasoconstrictor agents could be counterbalanced by either the activation of voltage-sensitive Ca+2 channels or by the induced gene transcription for vascular reagents, consequently the augmented mRNA for AGII. In addition, the cytoprotective effects through Bcl-xl protein family activation or PARP and iNOS

inhibition could point the attention on the use of rhEPO as a potential coadjuvant in the therapy of the septic shock (Fig. 11).

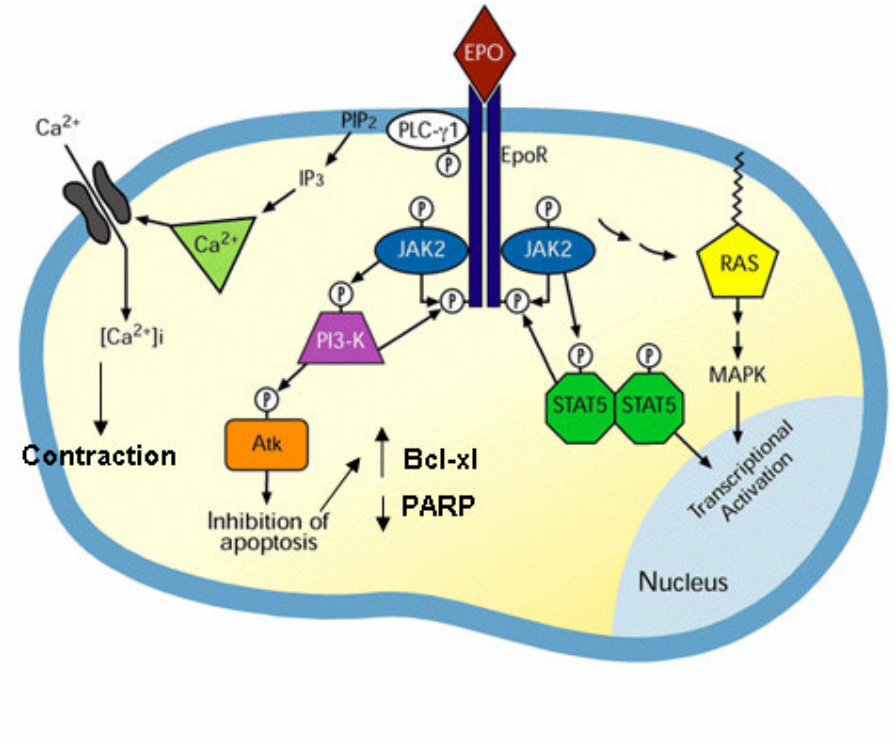

Fig.11 Mechanism by which rhEPO exerts beneficial vascular effect in an animal model of septic shock

References

1. American College of Chest Physicians/ Society of Critical Care Medicine Consensus Conference. Definition for sepsis and oragn failure and guidelines for the use of innovative therapies in sepsis. *Crit. Care Med.* 1992; 20: 864-874
2. Marshall JC, Cook DJ, Christou NV, et al. Multiple organ dysfunction score: a reliable descriptor of a complex clinical outcome. *Crit. Care Med*. 1995; 23: 1638-1652
3. Kang YH, Lee CH, Monroy RL, Dwivedi RS, Odeyale C, Newball HH. Uptake, distribution and fate of bacterial lipopolysaccharides in monocytes and macrophages: an ultrastructural and functional correlation. *Electron Microsc Rev*. 1992; 5(2): 381-419
4. Boveris A., Alvarez S., Navarro A. The role of mithocondrial nitric oxide synthase in inflammation and septic shock. *Free Radical Biol and Medicine* 2002. 9(1): 1186-1193
5. Zimmerman JJ. Defining the role of oxyradicals in the pathogenesis of sepsis. *Crit Care Med* 1995; **23**: 616–17
6. Noel RF, Sato TT, Mendez C, Johnson MC, Pohlman TH. Activation of the human endothelial cells by viable or heat-killed gram negative bacteria requires soluble CD14. *Infect. Immun*. 1995; 63: 4046-4053
7. Jelkmann W. Molecular Biology of Erythropoietin. *Internal. Medicine* 2004 43 (8): 649-659
8. Zhu H, Jackson T and Bunn F. Detecting and responding to hypoxia. *Nephrol Dial Transplant*. 2002, 17:3-7
9. Koury ST, Koury MJ, Bondurant MC, Caro J, Graber SE. Quantitation of erythropoietin-producing cells in kidneys of mice by in situ hybridization: correlation with hematocrit, renal erythropoietin mRNA, and serum erythropoietin concentration. *Blood* 74:645-651, 1989.
10. Klinglumuller U. The role of tyrosine phosphorilation in proliferation and maturation of erythroid progenitor cells-signals emanating from the erythropoietin receptor. Eur J Biochem 249: 637-647, 1997.
11. Constantinescu SN, Huang LJ, Nam H, Lodish HF. The erythropoietin receptor cytosolic juxtamembrane domain contains an essential, precisely oriented, hydrophobic motif. Mol. Cell 7: 377-385, 2001
12. Erbayraktar S, Yilmaz O, Gokmen N, Brines M. Erythropoietin is a multifunctional tissue-protective cytokine *Curr Hematol Rep*. 2003 Nov; 2(6):465-70.
13. . Bertok L. Endotoxins and endocrine system. Domest Anim Endocrinol 1998: 15(5): 305-8
14. . Jelkmann W. Use of recombinant human erythropoietin as an anti-anemic and performance enhancing drug. *Curr Pharm Biotechnol* 1: 11-31, 2000
15. Anagnostou A et al., *PNAS*, 1994
16. Bode-Boger SM, Boger RH, Kuhn M, Radermacher J, Frolich JC. Recombinant human erythropoietin enhances vasoconstrictor tone via endothelin-1 and constrictor prostainoids. *Kidney Int*. 1996;50(4):1255-61
17. Vogel V, Kramer HJ, Backer A, Meyer-Lehnert H, Jelkmann W, Fandrey J. Effects of erythropoietin on endothelin-1 synthesis and the cellular calcium messenger system in vascular endothelial cells. *Am J Hypertens*. 1997; 10(3): 289-96
18. Barrett JD, Zhang Z, Zhu JH, Lee DB, Ward HJ, Jamgotchian N, Hu MS, Fredal A, Giordani M, Eggena P. Erythropoietin up-regulates angiotensin receptors in cultured rat vascular smooth muscle cells. J Hypertens 1998, 16(12 Pt 1): 1749-57
19. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF. Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-XL induction. *Cell* 1999; 98(2): 181-91
20. Abdelrahman M, Sharples EJ, McDonald MC, Collin M, Patel NS, Yaqoob MM, Thiemermann C. Erythropoietin attenuates the tissues injury associated with haemorrhagic shock and myocardial ischemia. *Shock.* 2004; 22(1): 63-9
21. . Squadrito F, Altavilla D, Squadrito G, Campo GM, Arlotta M, Quartarone C, Saitta A, Caputi AP. Recombinant erythopoietin inhibits iNOS activity and reverts vascular dysfunction in splanchnic artery occlusion shock. Br J Pharmacol 1999, 127(2): 482-8
22. . Liu X, Xie W, Liu P, Duan M, Li W, Xu J. Mechanism of the cardioprotection of rhEPO on suppressing the inflammatory response in ischemia-repurfusion. *Life Sci*. 2006; 78(19): 2255-64
23. . Tong q, Chu X, Cheung JY, Conrad K., Stahl R, Barber DL, Mignery G, Miller BA. Erythropoietin-modulated calcium influx through TRP C2 is mediated by phopholipase C gamma and IP3R. Am J Physiol Cell Physiol 2004, 287(6): C1667-78

24. . Evgenov OV, Liaudet L. Role of nitrosative stress and activation of poly(ADP-ribose) polymerase-1 cardiovascular failure associated with septic and haemorrhagic shock. *Curr Vasc pharmacol* 2005, 3(3): 293-9

25. Assandri R, Egger , gassmann M, Niggli E, Baeur C, Forster I, Gorlach A. Erythropoietin modulates intracellular calcium in a human neuroblastoma cell line. *J Physiol* 1999, 15; 516(Pt2): 343-52

26. . Tong Q, Chu X, Cheung JY, Conrad K, Stahl R, Barber DL, Mignery G, and Miller BA. Erythropoietin-modulated calcium influx through TRPC2 is mediated by phospholipase Cγ and IP3R *Am J Physiol Cell Physiol* 287: C1667-C1678, 2004

27. . Mason-Garcia M, Clejan S, Tou JS, and Beckman BS. Signal transduction by the erythropoietin receptor: evidence for the activation of phospholipases A_2 and C. *Am J Physiol Cell Physiol* 262: C1197–C1203, 1992

28. . Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ, Neel BJ, Birge RB, Fajardo JE, Chou MM, Hanafusa H, Schaffhausen B, and Cantley LC**.** SH2 domains recognize specific phosphopeptide sequences. *Cell* 72: 767–778, 1993.

29. . Villalobos-Molina R, Ibarra M. Increased expression and fuction vascular alpha 1D-adrenoceptors may mediate the pro-hypertensive effects of angiotensin II. Mol. Interv. 2005, 5(6):340-2

30. . Krantz SB: Erythropoietin. *Blood* 77: 419-434, 1991

31. . Akimoto T, Kusano E, Inaba T, Iimura O, Takahashi H, Ikeda H, Ito C, Ando Y, Ozawa K, Asano Y. Erythropoitin regulates vascular smooth muscle cell apoptosis by a phosphatidylinositol 3 kinase-dependent pathway. *Kidney International* 58: 269-282, 2000

32. . Leverrier Y, Thomas J, Mathieu AL, Low W, Blanquier B and Marvel J. Role of PI3-kinase in Bcl-X induction and apoptosis inhibition mediated by IL-3 or IGF-1 in Baf-3 cells. Cell Death Differ. 6: 290-296, 1999.

33. . Chong ZZ, Li F, Maiese K. Erythropoietin requires NF-kappaB and its nuclear translocation to prevent early and late apoptotic neuronal injury durino beta-amyloid toxicity. *Curr Neurovasc Res* 2005, 2(5): 387-99.

34. . Sun Y, Jin K, Xie L, Childs J, Mao XO, Logvinova A and Greenberg DA. VEGF-induced neuroprotection, neurogenesis and angiogenesis after focal cerebral ischemia. *J Clin Invest* 2003, 111: 1843-1851.

Chapter 5

Effect of hydrogen sulphide and L-Cysteine on aorta and mesentery bed from streptozotocin-induced diabetic rats

5.1 Introduction

Diabetes mellitus is one of the most severe metabolic syndromes in our society, as it is associated with increased cardiovascular risk factors. The clinical complications can be broadly divided into microvascular disease, such as diabetic retinopathy and diabetic nephropathy, and macrovascular disease, such as accelerated atherosclerosis and hypertension, which are the main cause for morbidity and premature mortality among diabetic patients.

Diabetic vascular disease is accompanied by decreased formation of vasodilators, such as nitric oxide (NO) and prostacyclin, and increased formation of vasoconstrictors, such as eicosanoids and endothelin, which exacerbate the progression of vascular complications.

The exposure of endothelial cells to high glucose and lipids levels, typical of the diabetes, leads to the increased formation of reactive oxygen species which interfere with the synthesis, diffusion and action of NO on target cells. NO is the primary vasodilator mediator that regulates smooth muscle tone and an impaired function can induce to high peripheral vessel resistance and consequent high blood pressure.

In diabetic endothelial cells, oxidants can originate from mitochondria, NADPH oxidase or endothelial nitric oxide synthase (eNOS). These oxidants inhibit the endothelial function impairing the synthesis of NO by eNOS uncoupling, which can generate superoxide anion ($O_2^{\cdot-}$) in addition to NO. The reaction between NO and $O_2^{\cdot-}$ is the real 'key player' as it is very rapid and, not only impairs the diffusion of NO to target cells but also forms the reactive product peroxynitrite ($ONOO^-$), which reacts with proteins, lipids and DNA, reducing cell vitality. Furthermore, $ONOO^-$ inactivates prostacyclin synthase leading to the accumulation of inflammatory and prothrombotic eicosainoids, a further risk for increased blood pressure.

Recently, great attention has been focused on the activity of hydrogen sulphide (H_2S), always considered like a toxic gas with a typical 'gas rotten eggs' smell. It has a potential physiological and pathophysiological significance in the cardiovascular system.

H_2S is endogenously generated via both enzymatic and non enzymatic pathways (1). The enzymatic production is catalyzed from L-cysteine by cystathionine β-synthase (CBS) or cystathionine γ-lyase (CSE) (2). These two enzymes are tissue specific (1) and in particular, CSE is expressed more in the cardiovascular system (3).

Zhao et al. (3) demonstrated that H_2S induces vasodilation in the pre-contracted aorta and mesenteric arterioles *in vitro* in a rat model. The vasorelaxant effect could be ascribed to the membrane hyperpolarization consequence of the activation of the potassium ATP-dependent channels (K_{ATP}) on the smooth muscle cells (3). K_{ATP} channels can play an important role for the modulation of the vascular tone. In addition, the coadministration of apamin and charibdotoxin, calcium-dependent potassium channels inhibitors (K_{Ca}), reduced the vasorelaxant effect of H_2S on intact endothelium rat mesenteric artery (6), suggesting a double target for H_2S: K_{ATP} on the smooth muscle and K_{Ca} on the endothelium. K_{Ca} are activated by the endothelial hyperpolarizing factor (EDHF), but the relationship between H_2S and EDHF is not clear yet.

Another controversy is the interaction between NO and H_2S. Recent studies revealed that sodium hydrogen sulphide (NaHS), an H_2S donor, can enhance NO donors' activity on the rat aortic rings (7), even though it is well known that thiol groups can interact with nitric oxide and lead to the formation of nitrosothiols (8), which enable the biological activity of NO.

In a rat model of hypertension, induced by the administration of L-NAME, CSE expression and activity were reduced, presuming that NO may negatively regulate the endogenous levels of H_2S (4). Many hypotheses have been postulated but the NO and H_2S 'cross talk' needs to be further investigated.

5.1.2 Aim of the study

The aim of this study was, firstly, to understand the role of the endothelium and the implication of nitric oxide in the H_2S-induced vasorelaxation. Secondly, we tried to evaluate any difference in the activity of NaHS and L-cysteine, its endogenous precursor, on the aorta and mesenteric bed of diabetic rats compared to control rats.

5.2 Materials and methods

Male Wistar rats (Charles River) were used for *in vivo* and *ex vivo* experiments. Animals were housed in plastic cages and maintained in a light, humidity and temperature controlled environment. Food and water were allowed *ad libitum*. A period of seven days was allowed for rats acclimatization before any type of manipulation was undertaken. Animals (200-220g) were randomly divided in two groups, control (CTR) and streptozotocin-induced diabetic rats (STZ). Streptozotocin (STZ, 60mg/kg; Sigma Aldrich) or the vehicle (50mM sodium citrate buffer, pH=4.5) was administered intravenously, after animals' exposure to isofluorane (Abbott).

Animals were sacrificed after three weeks STZ injection, time in which diabetes was experimentally considered established. Diabetes induction was evaluated through an Accu-Chek monitoring system (Roche Diagnostics, USA) for the glycaemia measurement.

Animals were anesthetised using isoflurane and sacrificed by cervical dislocation and exanguinated.

We utilized thoracic aorta and mesenteric bed for *ex vivo* experiments.

5.2.1 Aorta experiments

Thoracic aorta was excised and cleaned of adherent connective tissue. It was cut into rings of ~3 mm length and mounted in organ bath, previously oxygenated and kept constantly under 37ºC.

Aortic rings were placed in a 2.5 ml organ bath containing Krebs' solution composed of (in mM) NaCl, 115.3; KCl, 4.9; $CaCl_2$, 1.46, MgSO4, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; and glucose 11.1; warmed at 37 °C, oxygenated (95% O2 and 5% CO_2), and connected to an isometric force transducer (model 7002, Ugo Basile,

Comerio) under a resting tension of 0.5 g. Changes in tension were recorded continuously by a polygraph (GraphTec Linearcorder). After about 60 min equilibration period, tissue was contracted with phenylephrine (PE, 1μM), an adrenergic α1-agonist, and the presence of a functional endothelium was verified by evaluating the relaxation to a dose-response curve of acetylcholine (Ach, 0.01-3μM). At the same time, aorta was endothelium denuded by using a wire, which filled perfectly the aorta diameter. The absence of the endothelium was evaluated by Ach (3μM) administration on a PE-induced stable tone.

NaHS and L-cysteine (L-Cys) were injected in a dose-curve response (10μM-10mM) on a PE-induced stable tone either in the presence or absence of endothelium. NG-nitro-L-arginine methyl ester (100μM, L-NAME), irreversible nitric oxide synthase (NOS) inhibitor, was administered on the basal tone for 20 minutes before PE administration.

5.2.2 Mesenteric Bed Experiments

The animals were sacrificed as previously described and the abdominal skin was cut along the *linea alba.* After the exposure of the entire gut, the mesenteric vein and artery were isolated and cleaned of adherent tissues. The artery and the vein were cannulated using an 18 gauge cannula. The artery cannula was directly connected to a pump able to flutter a solution of heparin 10 U/ml at 5 ml/min, in order to clean and avoid thrombi. The mesenteric bed was then connected to a pressure transducer in order to evaluate changing in perfusion pressure after different drugs administration. The mesentery was perfused at 2 ml/min speed by a Krebs' solution, continuously oxygenated and medicated by indomethacin (INDO, 10μM), a cycloxigenase inhibitor, to reduce the high production of released prostaglandins that affect the vascular tone. After about 20 minutes of equilibration period,

methoxamine (MTX, 10μM), an adrenergic α1-agonist, was added to the Krebs' solution. The endothelium integrity was evaluated by a *bolus* of Ach (10-100 pmol). NaHS or L-Cys (10μM-10mM) was infused at a 50μl/min and for 10 and 30 minutes, respectively. These time points were chosen from preliminary experiments, further described in literature (1). In the same way as the aortic rings, L-NAME (100μM) was administered by perfusion on a stable tone of MTX.

5.2.3 Chemical compounds

NaHS (Sigma Aldrich) is a salt capable to release HS^- and H_2S in solution, as described by the reaction:

$$NaHS + H_2O \rightleftharpoons H_2S + NaOH$$

L-Cys the metabolic substrate for Cystathionine β-synthase (CBS) and Cystathionine γ-lyase (CSE) was obtained from Sigma Aldrich. The final endogenous product of its catabolism is H_2S, which explicates its effect in a site specific way. All salts used for Krebs' solution were from Carlo Erba (Italy). U46619 and hET-1 were performed by Tocris (Italy), whereas Ach and PE were from Sigma Aldrich.

5.2.4 Statistical Analysis

The results are expressed as mean±s.e.m. and analysed using the two way ANOVA, followed by the Bonferroni post test, or the unpaired Student's t test. We considered significant P values less than 0.05.

5.3 Results:

5.3.1 Aorta-NaHS

NaHS explicated a concentration-dependent relaxation on *prior* pre-contracted aortic rings. The vasodilation was significantly higher in the aortic rings of diabetic rats (STZ group) ($P<0.0001$, Fig 1A) compared to the control (CTR group).

To investigate the possible involvement of nitric oxide (NO) on H_2S-mediated relaxation, we administered L-NAME, a NOS inhibitor, on the basal tone for 20 minutes before contracting the aortic rings. NaHS vasodilation was not altered by the NOS inhibition in the CTR and STZ group (Fig.1B, C) but the comparison between the two above groups still revealed a significant relaxation in the diabetic group at the higher concentration (10mM; Fig 1D).

Cheng and collaborators demonstrated that in the absence of a functional endothelium, H_2S induced a reduced relaxation in the mesenteric bed (6) of non-treated rats. In the same way, we tried to test whether the endothelium could affect NaHS dilation.

Interestingly, the relaxation was not altered by the removal of the endothelium either in the CTR or in the STZ group (Fig. 2A, B), but the comparison between the two groups without endothelium, still revealed a significant ($P<0.05$, Fig 2C) relaxation in diabetic than control aortic rings.

Interestingly, we also observed a double activity on a stable tone of PE, an effect which has never been reported before. NaHS was able to induce a contractile effect followed by vasodilation. This effect was observed especially at lower concentrations (10-100μM) and in both groups of animals (Fig 3A).

The removal of the endothelium tended to reduce (Fig. 3B, C) the above described effect in a significant manner in the CTR and in the STZ group ($P<0.005$, $P<0.05$, respectively).

To test whether this effect was an intrinsic capability of the sodium sulfide, we administered a dose-response curve on the basal tone of the aortic rings. No activity was observed either in the CTR group or in the STZ aortic rings.

The next step was to evaluate the possible involvement of NOS on the contractile effect. L-NAME treatment did not modify the contractile NaHS effect in the control group whilst it reduced ($P<0.05$) the contraction in the diabetic rats at 100μM (Fig. 4A, B).

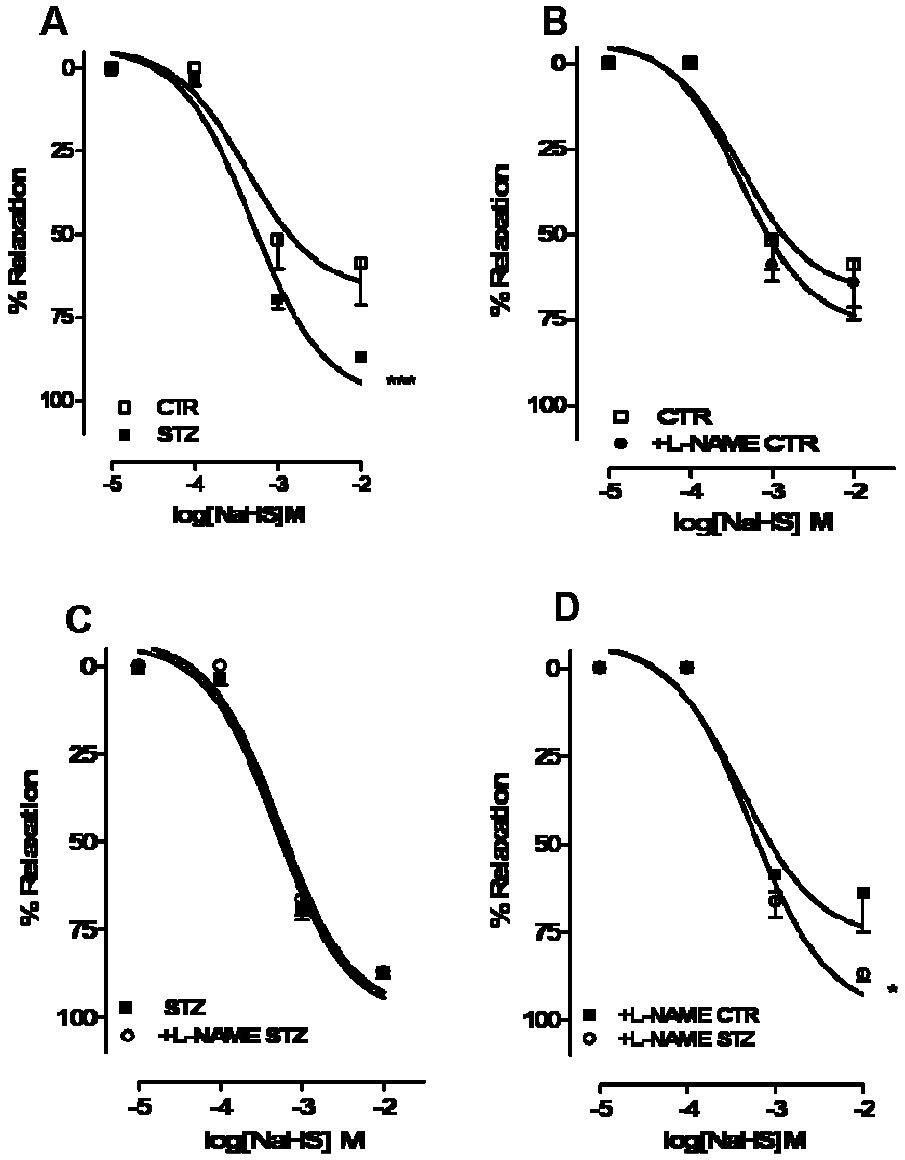

Fig.1 The NaHS-mediated relaxation was more potent in the diabetic rats than the control (A) (***P<0.0001). L-NAME did not modify the relaxation either in the CTR (B) or in the STZ group (C), in fact there was still a more potent (*P<0.05 *vs* +L-NAME CTR) relaxation in the diabetic group.

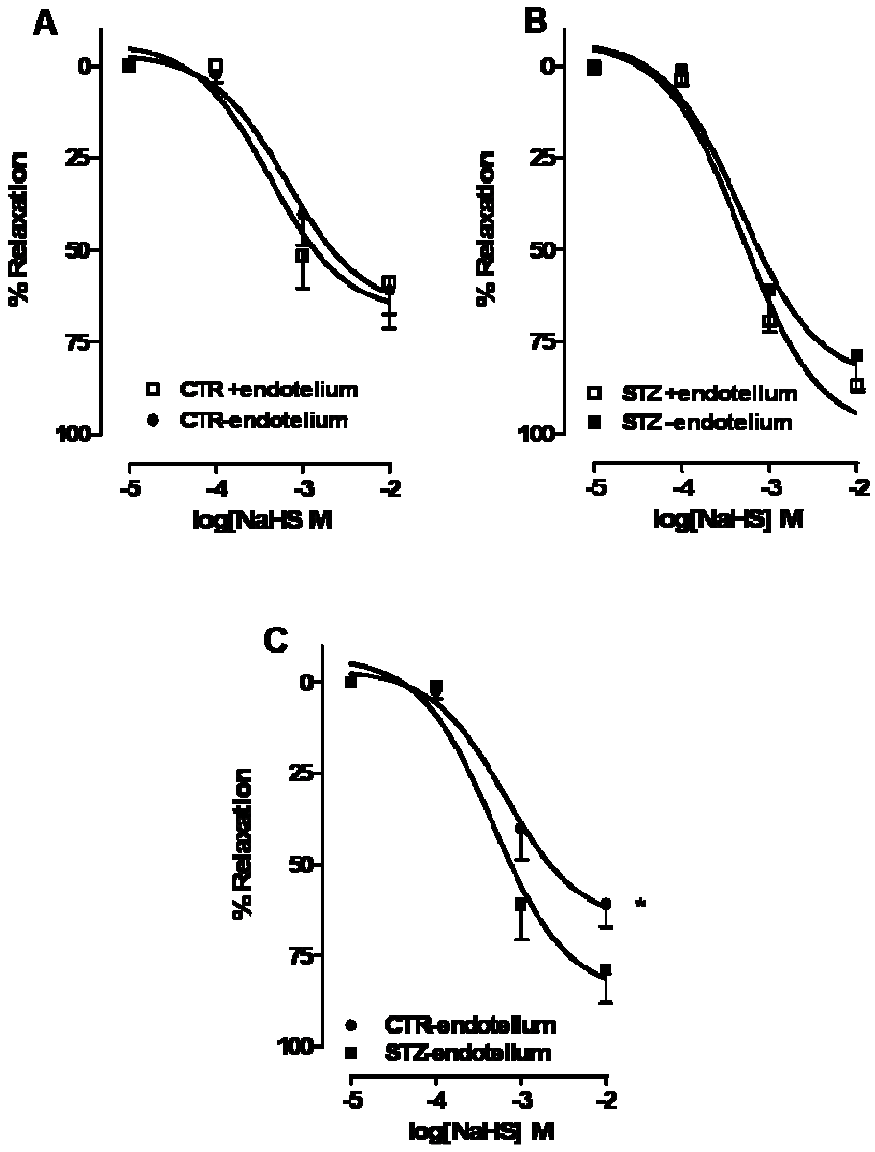

Fig. 2 The relaxation was not altered by the removal of the endothelium either in the CTR (A) or in the STZ group (B). The comparison between the two groups still revealed a significant (*P<0.05 *vs* CTR) activity on the diabetic aortic rings (C).

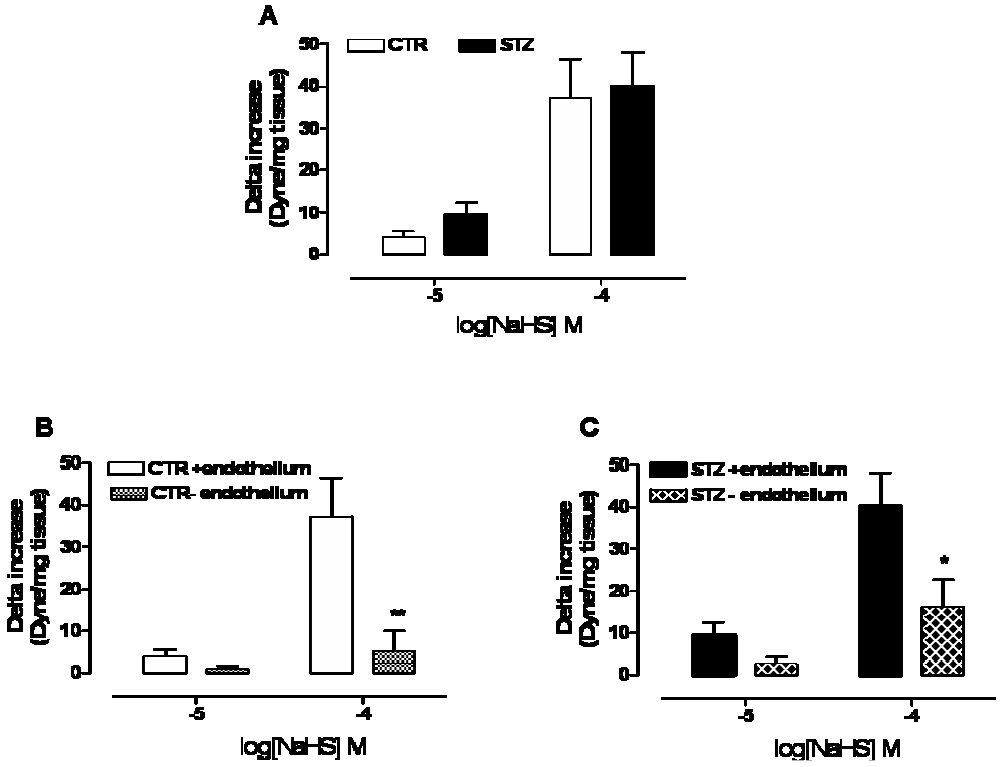

Fig. 3 Either the CTR or the STZ animals revealed a contractile effect on a stable tone of PE (A). The removal of the endothelium reduced NaHS-induced contractility in a significant manner (**P<0.005 *vs* CTR+endothelium) in the control group (B)and in the diabetic group (P<0.05) (C)

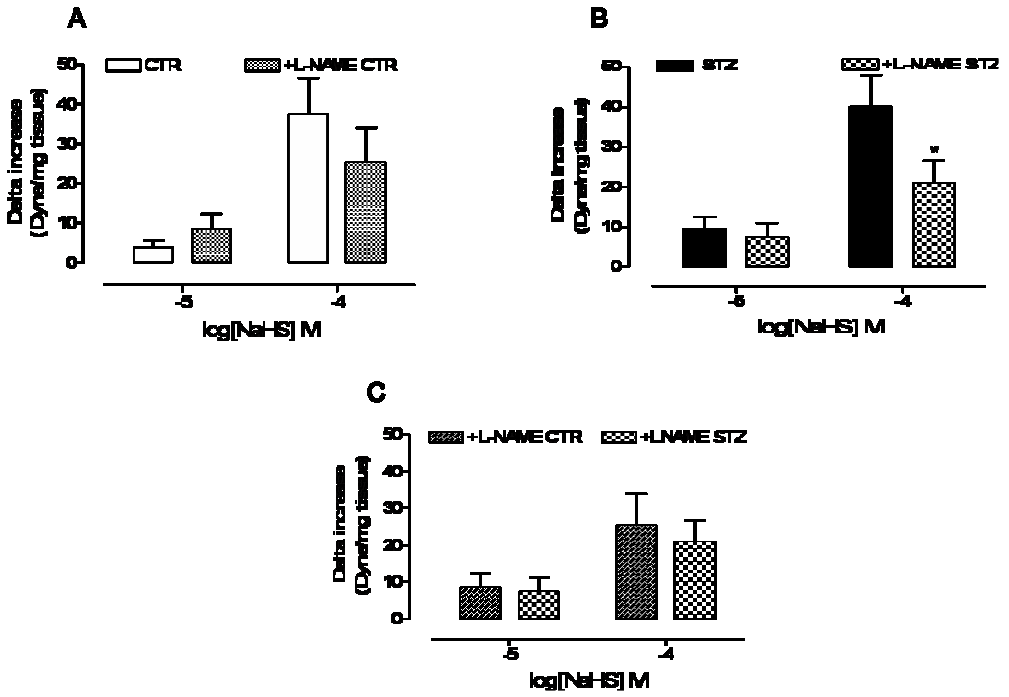

Fig. 4 L-NAME administration did not modify the contractile effect in the CTR (A) but slightly reduced it in the STZ group (*P<0.05) (B). The comparison between two groups plus L-NAME did not show any difference (C).

5.3.2 Aorta-L-Cysteine

In the same manner as NaHS, L-Cys induced a concentration-dependent relaxation in the aortic rings of the diabetic and control group. Its effect is more noticeable at higher concentrations (1-10mM) than NaHS.

There was no significant difference in the relaxation between the treated and non-treated group of animals (Fig.5A).

The addition of L-NAME on the basal tone for 20 minutes before PE did not affect L-Cys vasodilation either in the CTR or STZ rats (Fig. 5B, C).

To evaluate the effect of the endothelium on L-Cys vasodilation we used endothelium deprived aortic rings. The removal of the endothelium did not affect the relaxation in the CTR group (Fig. 6A), whilst it increased significantly the relaxation in the diabetic animals ($P<0.001$, Fig. 6B). Comparing the CTR and STZ endothelium-denuded aortic rings, L-Cys was still not able to induce a relaxation in the diabetic rings comparable to the control (1mM, $P<0.001$, Fig 6C).

As well as for NaHS, the contractile effect was observed after L-Cys administration too, on pre-contracted aortic rings of both groups (Fig.7A). Interestingly, following L-NAME administration, the contractile effect was increased significantly ($P<0.05$) at the concentration of 10μM in the control aortic rings (Fig.7B), whilst it was reduced significantly in the diabetic rings ($P<0.05$, Fig.7C).

The absence of a functional endothelium remarkably abolished the contractility in both the CTR and the STZ group (Fig. 8A, B).

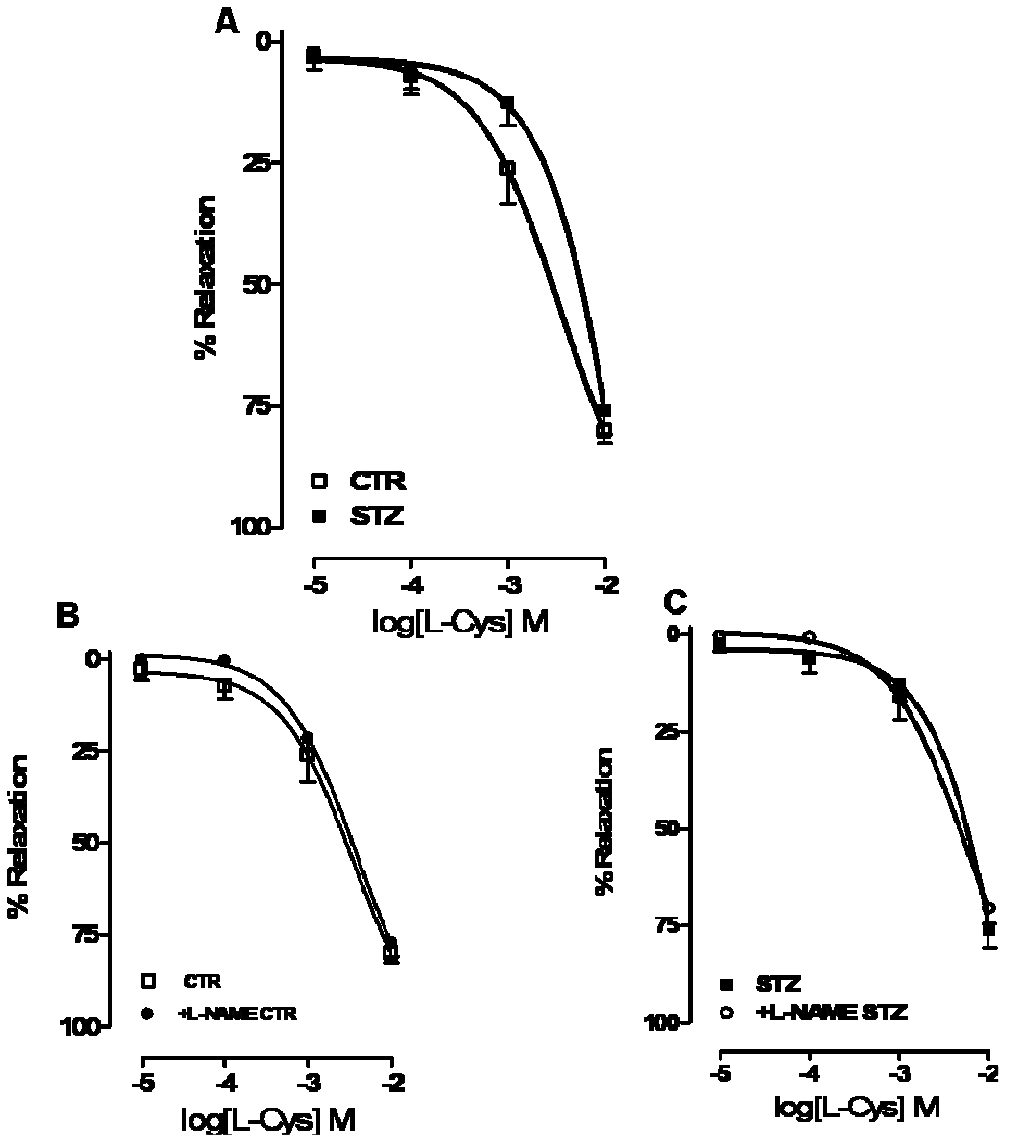

Fig. 5 There was no significant difference between the two groups of animals (A). L-NAME did not modify L-Cys-induced relaxation in the non-treated (B) and treated animals (C).

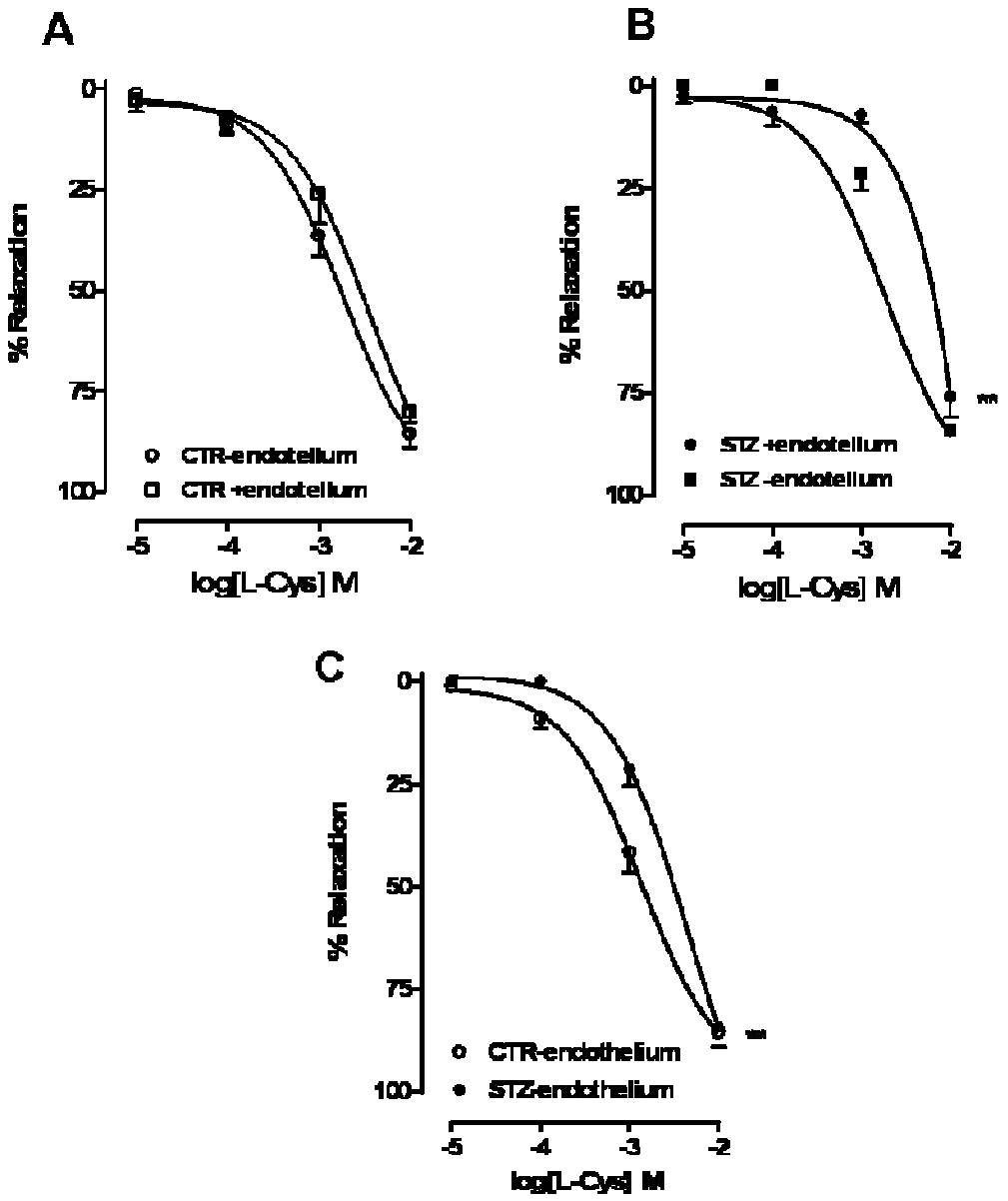

Fig. 6 The removal of the endothelium did not affect L-Cys-induced relaxation in the CTR (A) whilst it increased significantly the vasodilation in the STZ group (**P<0.001 *vs* STZ-endothelium) (B). The comparison with the CTR revealed a major effect on the STZ especially at 1mM concentration (**P<0.001 *vs* STZ) (C)

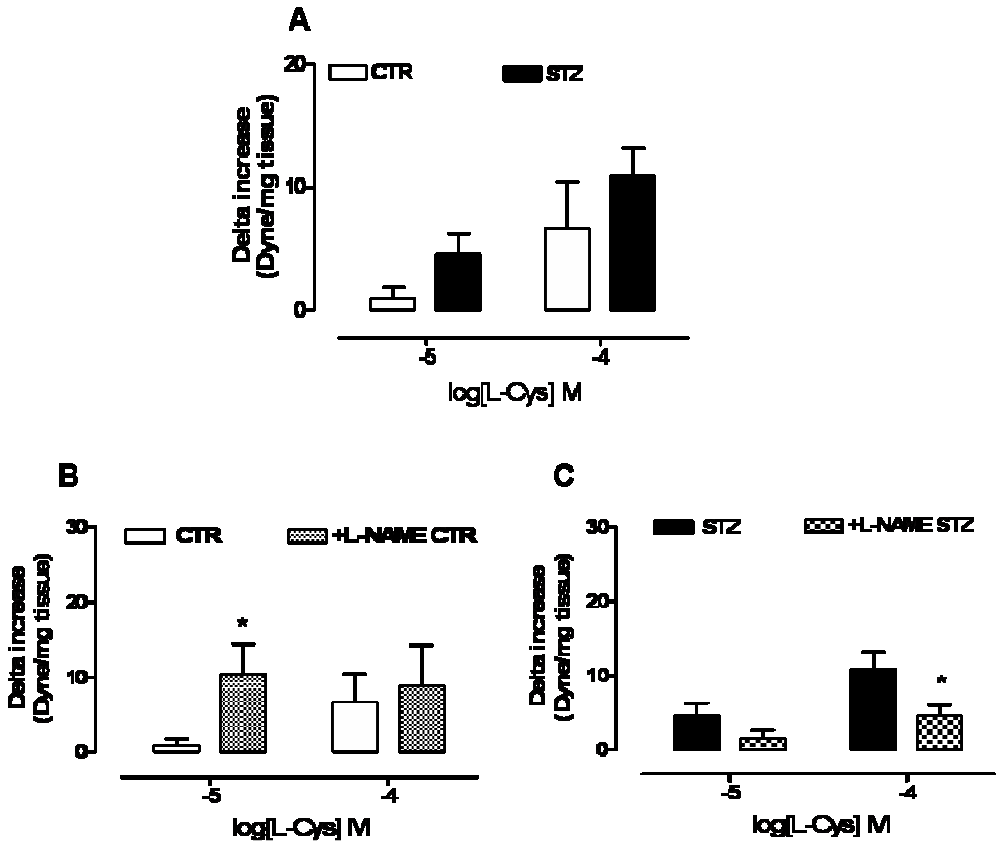

Fig. 7 There was no difference between CTR and STZ on the L-Cys-induced contraction (A), but the administration of L-NAME increased (*P<0.05 *vs* CTR) the contractility at 10µM in the CTR (B), and reduced (*P<0.05 *vs* STZ) it at 100µM in the STZ (C).

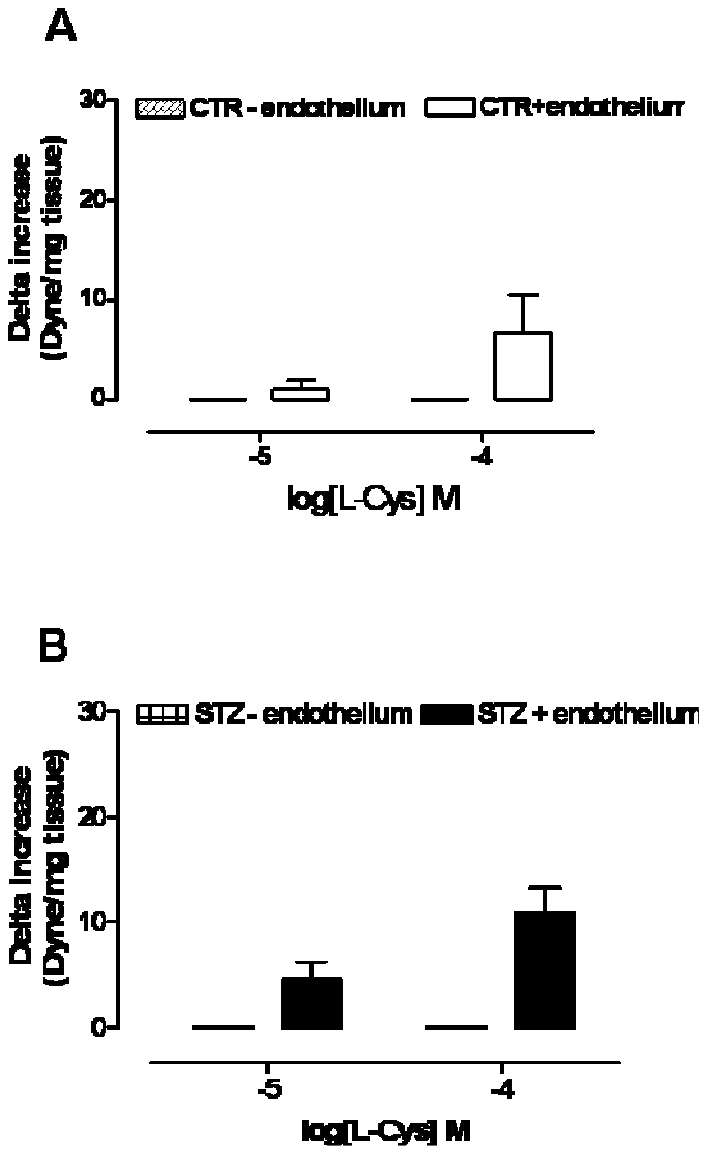

Fig. 8 The removal of the endothelium completely abolished L-Cys-induced contractility either in the CTR (A) or in the STZ (B).

5.3.3 Mesenteric bed-NaHS

Intravenous *bolus* of H_2S decreases blood pressure in a rat model *in vivo* (6). In our experimental conditions, *ex vivo*, NaHS was able to induce vasodilation of the mesenteric artery bed, decreasing the perfusion pressure. The treatment with NaHS did not reveal any substantial difference between CTR and STZ groups (Fig. 9A).

The involvement of NO on the induced vasodilation was evaluated by perfusing the mesenteric bed with L-NAME.

Interestingly, L-NAME perfusion increased NaHS-mediated vasodilation either in the diabetic group or in the control group (Fig. 9B, C). This effect was more potent at lower concentrations (0.1-1 mM) in the diabetic animals and at higher concentrations (1-10mM) in the control animals. Comparing the same data, NaHS induced a higher relaxation ($P<0.05$, Fig. 9D) in the diabetic mesentery than the control, under L-NAME perfusion.

As observed for the aorta, these compounds at lower concentrations (10-100μM), were able to induce an increase of the perfusion pressure on the MTX-induced stable tone followed by a vasodilation in the mesenteric bed (Fig. 10A).

L-NAME perfusion caused a lower contraction on the MTX-pre-contracted control mesentery bed ($P<0.05$, Fig. 10B) induced by NaHS but there was no significant effect noted on the diabetic mesentery (Fig. 10C).

Comparing the effect of L-NAME on the reduced NaHS contraction, there was no difference in the contractive force between diabetic and control mesentery, with the exception of 10μM ($P<0.05$, Fig. 10D) that was statistically reduced in STZ group.

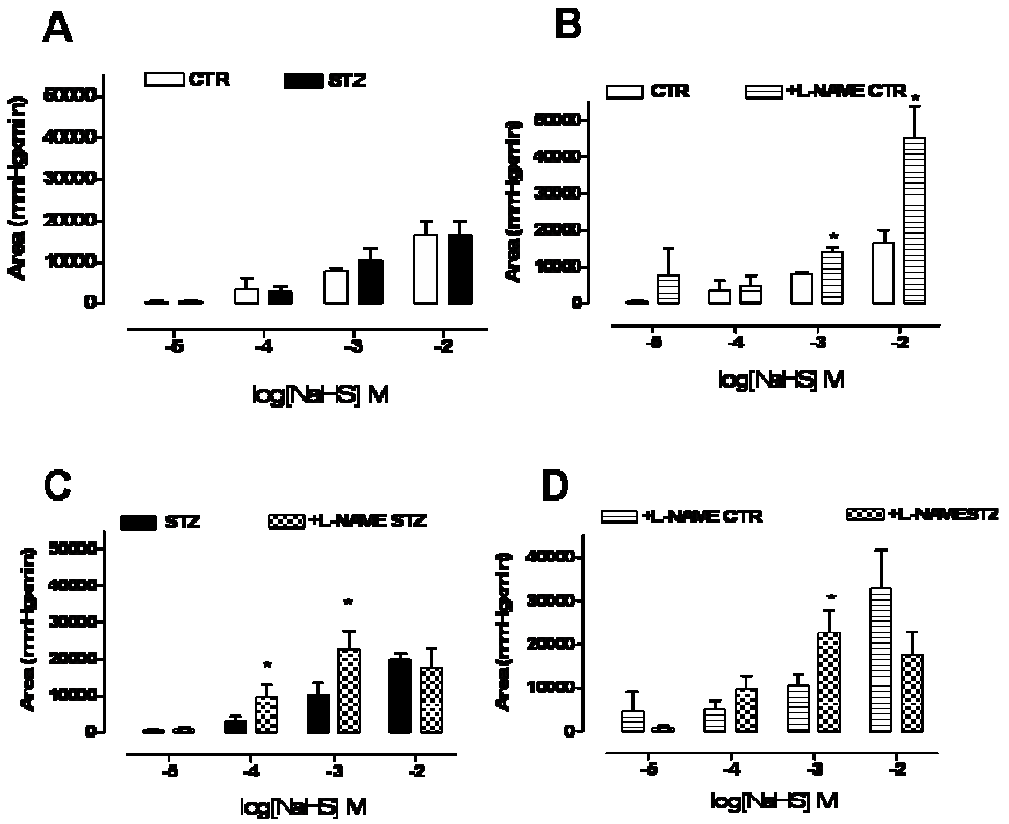

Fig. 9 There was no difference between CTR and STZ (A) in the relaxation in the mesentery but the administration of L-NAME increased the relaxation either in the CTR (B) (*P<0.05 *vs* CTR) or in the STZ (C) (*P<0.05 *vs* STZ). The comparison between the two groups (D) revealed a significant (*P<0.05) difference at 1mM in the STZ, under L-NAME treatment (D)

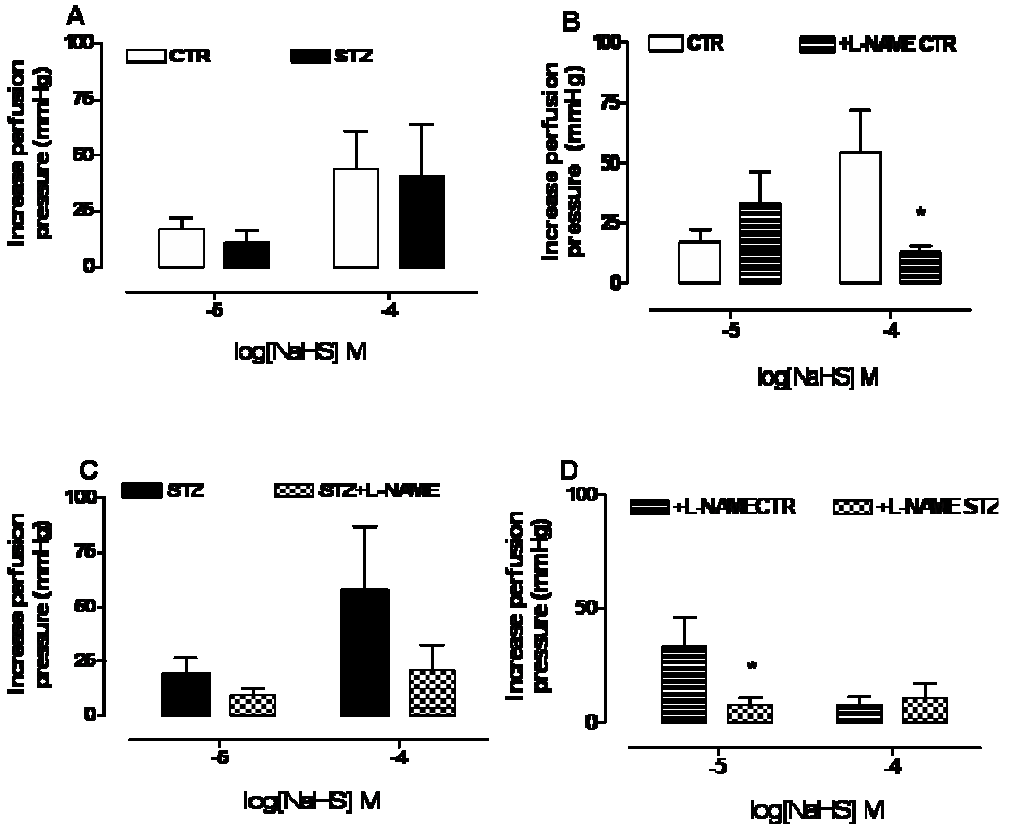

Fig. 10 NaHS-induced contraction on MTX did not reveal any difference between CTR and STZ (A) L-NAME reduced this effect at 100µM in the CTR (B) but not in the STZ (C). In the panel D, it is represented the comparison between +L-NAME CTR *versus* +L-NAME STZ; the effect was significantly lower at 10µM (*P<0.05)in STZ group.

5.3.4 Mesenteric bed-L-Cysteine

In the same way as the aorta, L-Cys induced a concentration-dependent relaxation, especially observed at the highest concentration in both groups of animals (Fig. 11A). The L-Cys effect on the perfusion pressure was significantly ($P<0.05$) higher in the diabetic mesentery at the concentration of 10mM compared to the control rats.

Nevertheless, it has to be noted that L-Cys explicated a more pronounced relaxation than NaHS especially at the higher concentration (10mM).

L-NAME treatment did not modify the relaxation in the diabetic mesentery (Fig. 11B) whilst it induced a more significant ($P<0.005$) dilation in the control (Fig. 11C) at 10mM. Comparing the same data, L-Cys-induced vasodilation was reduced significantly ($P<0.05$, Fig. 11D) in the diabetic *versus* control mesentery at the highest concentration, but not at the lower doses.

An increase in perfusion pressure effect, observed at lower concentrations, was observed also in mesenteric bed in both groups of animals (Fig. 12A), with an increased tendency for the diabetic to contract, even though the effect was not significant compared to the control group. Furthermore, L-NAME perfusion increased significantly ($P<0.005$) the contractile effect of L-Cys (100μM) in the control group (Fig. 12B) but not in the diabetic (Fig. 12C).

Comparing the same data, NOS inhibition enhanced the increase in perfusion pressure in the control in a significant manner (100μM; $P<0.05$), rather than in diabetic group (Fig. 12D).

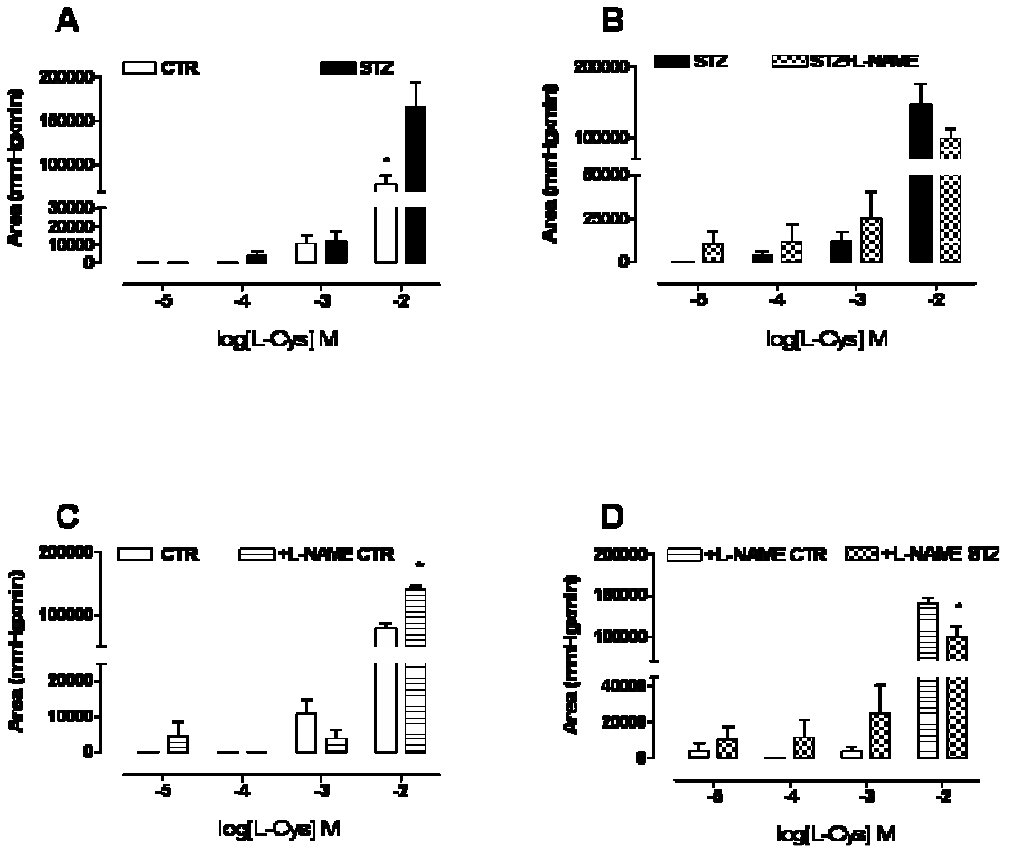

Fig. 11 L-Cys induced a significant (*P<0.05 *vs* CTR) increase in the relaxation in the STZ at the highest concentration (A). L-NAME treatment did not modify the relaxation in the STZ (B), whilst it increased the vasodilation (*P<0.05 *vs* CTR) at the dose 10mM in the CTR (C). The comparison between the two groups, under L-NAME treatment, revealed a significant (*P<0.05 *vs* CTR) CTR compared to the STZ (D).

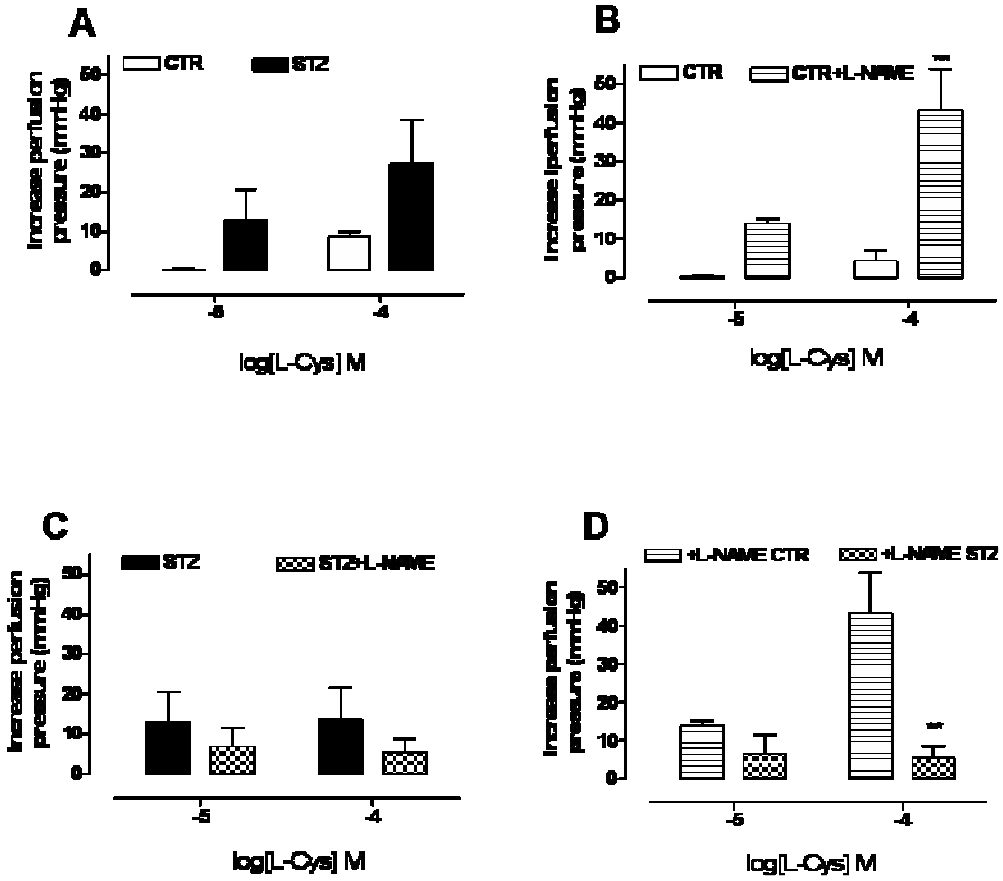

Fig. 12 L-Cys induced a contraction on a stable of MTX in the mesentery either in the CTR or in STZ (A). The administration of L-NAME increased (**P<0.005 *vs* CTR) the perfusion pressure in the CTR (B) but not in the STZ (C). The comparison between the two groups, under L-NAME treatment, still revealed a major effect in CTR group (**P<0.005 *vs* +L-NAME CTR).

5.4 Discussion

Nitric oxide has been highly studied over the last decades, but the discover of the 'third gastransimtter' opens new prospective for pathologic conditions like diabetes, where high blood pressure and the vascular consequences are the most deleterious side effects. Like NO and CO, H_2S can affect the biological structures and functions of the human body at molecular, cellular and tissue levels.

In the present study, as already demonstrated in literature, we show that both NaHS and L-Cys are potential vasodilators in the aorta and mesentery of non treated rats. The vasodilation resulted endothelium independent in the control aortic rings for both compounds.

In our experimental conditions, there was no interference between NO and H_2S, as previously described by Hosoki and collaborators (8), in the aorta. In contrast, in the mesenteric artery, L-NAME augmented the relaxation either after NaHS or L-Cys infusion.

The aorta is a model of capacitance vessel whilst the mesenteric bed represents a model of resistance vessels. The isolated and perfused rat mesenteric bed is an experimental model by which a drug can be proved to be effective on the regulation of peripheral resistance to blood flow and thus to blood pressure. We utilized this model to measure and better identify vascular reactivity of hydrogen sulphide in a peripheral resistance vascular district.

We can therefore, speculate that NO interferes in a negative way on the H_2S-induced relaxation in the mesenteric bed, possibly leading to the formation of nitrothiols, which oppose the biological gas effect.

The second aim of our study was to evaluate any difference between the physiological and the diabetic pathological *status*, and again try to understand the role of the endothelium and NO on H_2S pathway.

NaHS induced a more prominent relaxation in the diabetic aorta than the control, but the same effect was not observed with L-Cys treatment.

It has been demonstrated that K_{ATP} channels are the final target for H_2S activity (3) on the vascular smooth muscle cells. Under euglycemic conditions, K_{ATP} channels are maintained in an open state, resulting in a hyperpolarizing membrane potential (9). When glucose is elevated, ATP levels increase and displace bound ADP on K_{ATP} channels, resulting in channel closure. Closure of K_{ATP} channels depolarizes the membrane, allowing voltage-dependent Ca^{2+} channels to open (10) inducing to the typical increased blood pressure in the diabetes. Our findings in the diabetic aortic rings could suggest a major effect of the hydrogen sulphide on the K_{ATP} channels in the aorta, as they could be more susceptible to the opening in order to counterbalance the increased vascular hyperactivity, depolarization, typical of a diabetic *status* (11).

In contrast, the administration of L-Cys, a precursor metabolic product for hydrogen sulphide, induced a higher relaxation in the control rings than the diabetic rings. CSE, the enzyme that catalyzes the aminoacid metabolism in the vascular tissues, could be less active in the diabetic status, as described by Zhong (3), who supported a reduced enzyme reactivity *in vivo*, due to the high levels of NO, typical of endothelial damage in hypertension and diabetes.

Furthermore, the treatment with L-NAME did not reveal any difference in the NaHS or L-Cys-induced vasodilation in the aorta of both groups of animals. So, we could speculate that CSE is less active in the diabetic rats probably because of a

'cross talk' with endothelium derived factors, like endothelin, which could counterbalance the enzyme activity, and not NO, as confirmed either by the ineffectiveness of L-NAME on the relaxation or by the removal of the endothelium, which increased the relaxation in diabetic aortic rings after L-Cys, but not NaHS.

In the mesenteric bed, there was still no difference between the relaxation induced by NaHS in the CTR and STZ animals, but the addition of L-NAME increased the vasodilation in both groups.

In our experimental conditions, L-NAME treatment revealed the effect of EDHF, as indomethacin was added to the perfused Krebs' solution. EDHF, as already demonstrated by using another experimental diabetic induced method (12), and confirmed by our experiments, was less produced in the diabetic animals, consequence of an impaired endothelium.

EDHF hyperpolarizes the vascular smooth muscle through the activation of K_{Ca+2} channels; so, the increased relaxation, which was more visible in the diabetic at lower concentration of NaHS, could be explained by an induction of EDHF production by H_2S or by a synergy activity in the two compounds.

The same effect was not registered with L-Cys infusion to the mesenteric artery, confirming the critical role of the enzyme CSE in the diabetic status.

An innovative effect, ever registered, was the contractile capacity of these compounds on a stable phenilephrine tone. This effect was endothelium dependent either for L-Cys or for NaHS in the aortic rings, confirmed by the addition of L-NAME and the removal of the endothelium, which respectively decreased or completely abolished the contraction.

Gluais et al (11) demonstrated that in the aorta of SHR and WKY, the endothelium-dependent contractions elicited by A23187 and Ach involved the release of

thromboxane A2 and prostacyclin with a most likely concomitant contribution of PGH2. So, we could speculate that both NaHS and L-Cys could induce pro-contractile factors activity in the aorta and mesenteric bed. The implicated contractile factors could be either tromboxane A2 or endothelin, both endothelium derived. The involvement of endothelin seems to be more suitable in the case of the mesentery, as the prostaglandins synthesis was inhibited by the addition of indomethacin in the perfused solution.

In conclusion, further understanding of the underlying mechanism for H_2S could open future prospective for clinical trials in conditions like diabetes and hypertension. These pathologies are characterized by endothelial damage and high blood pressure, so the use of a probable H_2S donor could prove to be efficient, as it can either directly interact with the smooth muscle cell or can be produced at the level of the vascular smooth muscle (1). Nevertheless, more studies are required to elucidate its clinical contribution, especially for explaining the endothelium implication in the contractile activity.

References

1. Wang. Two's company, three's a crowd-can H2S be the third endogenous gaseous transmitter? *FASEB J* 16: 1792-1798, 2002

2. Bukovska G, Kery V, and Kraus JP. Expression of human cystathinine β-synthase in Escherichia coli: purification and characterization. *Protein Expr Purif* 5: 442-448, 1994

3. Zhao W, Zhang J, Lu Y and Wang R. The vasorelaxant effect of H2S as a novel endogenous gaseous KATP channel opener. The EMBO Journal 2001, 20 (21): 6008-6016

4. Zhong GZ, Chen FR, Cheng YQ, Tang CS, and Du JB. The role of hydrogen sulphide generation in the pathogenesis of hypertension in rats by inhibition of nitric oxide synthase. *Journal. of Hypert*. 2003, 21:1879-1885

5. Mok YYP, Atan MS, Ping CY, Zhong WZ, Bhatia M, Moochhala S and Moore PK. Role of hydrogen sulphide in haemorrhagic shock in the rat: protective effect of inhibitors of hydrogen sulphide biosynthesis. *British Journal of Pharmacology* (2004) 143, 881–889

6. Cheng Y, Ndisang JF, Tang G, Cao K, Wang R. Hydrogen sulfide-induced relaxation of resistance mesenteric artery beds of rats. Am J Physiol Heart Circ Physiol 2004 Nov; 287(5):H2316-23.

7. Padgett CM, Whorton AR. Regulation of cellular thiol redox status by nitric oxide. *Cell Biochem Biophys*. 1995;27(3):157-77

8. Hosoki R, Matsuki N, Kimura H. The possible role of hydrogen sulphide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem. Biophysyc. Research* 1997 237, 527-531.

9. Ashroft SJH, Aschcroft FM. Properties and functions of ATP sensitive K-channels. *Cells Signal* 2: 197-214, 1990

10. Arkhammar P., Nilsson T, Rorsman P, Berggren PO: Inhibition of ATP-regulated K+ channels precedes depolarization-induced increase in cytoplasmic free Ca2+ concentration in pancreatic β-cells. *J. Biol Chem* 262: 5448-5454, 1987

11. Su X, Changolkar A, Chako S, Moreland RS. Diabetes decreases rabbit bladder smooth muscle contraction while increasing levels of myosin light chain phosphorylation. *Am J Physiol Renal Physiol* 2004;287(4):F690-9.

12. Burnham MP, Johnson IT, Weston AH. Impaired small-conductance Ca(2+)-activated K(+) channel-dependent EDHF responses in Type II diabetic ZDF rats. *Br J Pharmacol* 2006;148(4):434-41.

13. Gluais P, Yang D, Zhang JN, Vanhoutte PM, Feletou M. *Fundam Clin Pharmacol* 2004;18(3):321-6

II. Discussion

Sepsis and diabetes are two different cardiovascular diseases. Sepsis is an infective pathology whilst diabetes is a metabolic syndrome . The common underlying matrix is the effect on the cardiovascular system. Sepsis can be caused by the infection of a bacteria, which invades organs like the lung, and then dissolve to the circulation promoting a systemic inflammatory process, leading to a massive hypotension; whereas, diabetes is promoted by the absence or the inefficency of insulin, occurring hypertension and atherosclerosis as secondary effects, which are the main responsible of diabetes-induced mortality in the patient. Thus, on one hand, sepsis promotes a severe vasodilatation with low blood perfusion and consequently organ injury and on the other hand, diabetes promotes profound vasoconstriction, inducing hypertension.

The systemic inflammatory pattern, observed in sepsis, is directly activated by the presence of a pathogen agent that invades the organism. The recognition of the pathogen by the innate immune system leads to the activation and interaction of a number of effective cascades such as the complement, TLRs pathway, coagulation, bradykinin/kinin and hematopoietic systems, that can interact with each other and facilitate the release of a myriad of mediators in the acute phase response, such as eicosanoids, cytokines, chemokines, adhesion molecules, reactive free radicals, platelet-activating factor (PAF), and nitric oxide (NO). The macroscopical reflection of these pro-inflammatory patterns is the vascular dilation according to the elevated amount of NO, prostacyclin produced and increased activity of potassium ATP-dependent channels.

The most common infectious sources of severe sepsis are the lungs, abdomen, and urinary tract. In the Chapter 1, we studied the effect of Gram positive bacteria, like S.*aureus*, or Gram negative bacteria, like E.*coli*, on human pulmonary epithelial cell line. The stimulation of human airway epithelial A549 cells with bacteria induced the production of a chemokine CXCL-8, responsible of the recruitment of neutrophils. The costimulation with both type of bacteria, example of co-infection typical of sepsis, potentiated the amount of CXCL-8 detected. CXCL-8 overproduction was due to the activation of TLRs, innate immune receptors, which via NF-kB or AP-1, nuclear transcription factors, may mediate the induced synthesis for pro- and anti-inflammatory cytokines, and in particular of chemokines. The arrival of neutrophils in the site of the infection, one of the first step for the host immune defence, promotes the production of many anti-bacterial products, such as defensins and NO, to combat and promote the clearance of the pathogen. As the lung is enriched of vessels, the pathogen, that escapes the host defence, can migrate to the systemic circulation and evoke a more severe systemic inflammatory pattern.

Once in circulation, the pathogen can be recognised by immuno-competent circulating cells, like blood macrophages or monocytes, or even by tissue macrophages. The recognition of the infectious agent or of some of its structural components induces the production of many inflammatory mediators, like TNFα, IL-1β and IL-6. First of all, cytokines like TNFα, IL-1β and IL-6 can either act in an autocrine manner, activating the same cell by which they are produced, or in a paracrine manner, activating other immuno-competent cells. Other mediators produced in this pathological state can be IL-4, IL-2, IL-12 and so on, capable of inducing the differentiation and the proliferation of T and B lymphocytes, activating

the adaptive immune system. This is the link between the innate and the adaptive immune system.

Among the paracrine actions of macrophagic cytokines there is the activation of the stromal cells, like endothelial or vascular smooth muscle cells. These cells are able to 'sense' the pathogen and promote the synthesis of inflammatory mediators, in particular NO, by the inducible NOS isoform (iNOS). As observed in the Chapter 2, iNOS was induced by both Gram positive and Gram negative bacteria. The overproduction of NO has a double and ambiguous action. Macrophages-derived NO is essential for the host defence, capable of killing the bacteria through the 'suicide mechanism', which implicates either a cytostatic or cytotoxic effect (8). Indeed, large amount of NO cause autoinhibition of the mitochondrial respiration by inhibiting several key enzymes in the mitochondrial respiratory chain, like NADH-ubiquinone reductase or succinate-ubiquitinone oxidoreductase, and the Krebs' cycle, through the formation of radical species (1), which interfere with the biological vitality of the pathogen, leading to its clearance. Our experimental conditions revealed a higher production of NO by J774.2 macrophages stimulated with E.*coli* rather than S.*aureus*, which released significant lower amount of NO at 24 hours. This may be a possible explanation for a more threatening clinical septic condition after Gram positive bacteria challenge.

The ambiguous face of NO is that its effects can be traduced even on the host organism. NO is also produced by the vascular smooth muscle cells and endothelial cells, leading firstly to a profound vasodilatation, typical deleterious aspect of the septic shock, and secondly to the damage of cellular structures. Our results revealed a similar production of NO by vascular smooth muscle cells stimulated with E.*coli* and S.*aureus*. The vascular E.*coli*-induced NO production is well documented, but

the activity of S.*aureus* on vascular cells but not on macrophages may be the answer to the highest severity of Gram positive-induced sepsis, may be due to a high vascular response to the bacteria and a lower or later immuno-competent reaction.

Besides NO, other radicals (ROS) and oxidants can be produced by the recruited inflammatory cells. Consequently, ROS could interact with NO and form RNS that could cause damage at the double strand of DNA, peroxidation of lipids and nitration of proteins, especially at the level of tyrosine. The modification in the structural cellular physiology, in the same way as in the diabetes, can trigger the activation of the nuclear enzyme PARP-1. Activation of PARP-1 results in the rapid depletion of intracellular NAD+, its substrate, slowing the rate of glycolysis, electron transfer and ATP formation, which ultimately leads to cell death.

Interestingly, exogenous cell stimulation by ROS, like H_2O_2, as studied in the Chapter 3, activated monocytes or other immuno-competent cells to produce chemokines, like CXCL-8, and cytokines. The same pattern may reproduce in the host organism, so that ROS can induce the recruitment of other leucocytes and antigen presenting cells (macrophages or dendritic cells), further exacerbating the redundant situation in the inflamed site, already challenged by the bacteria invasion. The activation of TLR4 by E.*coli* or respective PAMPs, and of the heterodimer TLR2/1 by S.*aureus* and TLR2 ligands were responsible of the increased CXCL-8 release. Interestingly, the activation of TLRs did not only keep the inflammatory pattern active, but also induced the induction of an endogenous cytoprotective protein, Nrf-2. Nrf-2 is a nuclear transcription factor that can counteract the activity of the oxidative stress. Its translocation through the nucleus was endogenously followed the stimulation of THP-1 cells with N-acetylcysteine, a well-known anti-

oxidant agent. Surprisingly, the activation of TLR4 and, in particular, the heterocomplex TLR2/1 by specific ligands alone, still promoted the translocation of Nrf-2 to the nucleus, implicating the role of TLRs in this protective pathway. Furthermore, the co-stimulation with TLRs specific ligands *plus* H_2O_2 promoted a significant increase in Nrf-2 amount in the nuclear protein extract, especially after LPS and Pam3CSK4 coadministered with the ROS. It is known that TLRs signalling pathways achieve the activation of many nuclear transcription factors like NF-kB or AP-1, which in turn promote the mRNA transcription for pro-inflammatory cytokines and chemokines. So, as described in literature, the activation of TLRs is a sort of host defence and therefore requires the activation of destructive mediators for the pathogens. Hence, our results confirmed that, besides the invasive patterns adopted by the organism for struggling the invasion of pathogens with the deleterious consequences on the host, there are some pathways, like the Nrf-2, that may oppose via TLRs to the destruction of physiological mechanisms, in order to re-establish the entire immuno-competent or vascular system.

These results were related to a condition of oxidative stress followed the invasion of bacteria like S.*aureus* and E.*coli*, responsible of inducing the septic shock. In the case of diabetes, it was also demonstrated that oxidative stress is a serious and secondary mechanism consequence of the high blood pressure and then of the shear stress. Recently, Rossi et al. (2) revealed that the levels of stress sensitive enzyme heme oxygenase-1 (HO-1) were impaired, index of a prolonged inflammation in the diabetes. HO-1 is the inducible isoform of the first and rate-controlling enzyme of heme degradation. HO-1 is up-regulated by oxidative stress stimuli and has potent cytoprotective and anti-inflammatory functions via decreasing tissue levels of the

pro-oxidant heme along with production of bilirubin and the signalling gas carbon monoxide (3). It was also demonstrated that HO-1 could be induced by NO or PGJ2, increasingly produced during the diabetes, via Nrf-2 nuclear translocation in the RAW264.7 murine macrophages (4).

Therefore, the inflammatory process, which is at the basis of both pathologies considered in this thesis, could be opposed by the therapeutic induction of endogenous cytoprotective mechanisms, like Nrf-2 or the effector HO-1, in order to at least, combat the oxidative stress.

A further aspect considered in my PhD training, was the effect on the vessel wall by the sepsis and diabetes. As described above, the massive vasodilatation in the sepsis is due to the inappropriate activation of vasodilator mechanisms. The overexpression of iNOS either by macrophages or by vascular smooth muscle cells and endothelial cells leads to an overproduction of NO. It can promote vasodilatation by the activation of the soluble guanilate cyclase with a consequent formation of cGMP that activates a cGMP-dependent protein kinase (PKG), the responsible kinase of the myosin light chain phosphorilation in the vascular smooth muscle cell.

Furthermore, NO can also activate the potassium ATP-dependent channels (K_{ATP}), via a cGMP-dependent mechanism (5), and calcium-dependent potassium channels (K_{Ca+2}), via a direct nitrosylation of the channel and activation of protein kinase G (PKG) (6, 7), leading to a profound vascular relaxation.

Chapter 4 describes the study of a possible coadjuvant for sepsis therapy, human recombinant erythropoietin (rhEPO). rhEPO treatment increased either the low blood pressure typical of septic rats *in vivo* or the vascular hyporeactivity to vasoactive agents like phenilephrine, endothelin, tromboxane and angiotensin II *in*

vitro. The endothelium impairment was diminished, confirmed either by the increased acetylcholine-induced relaxation on a stable tone of phenilephrine, or by the reduction of iNOS and ICAM-1 protein expression in the aorta. rhEPO also revealed antiapoptotic activity by increasing the amount of Bcl-2 and Bcl-xl, antiapoptotic proteins, and decreasing the value of PARP-1, enzyme able to induce to the DNA repair or to the cell death (apoptosis). Therefore, rhEPO may have all the characteristics to be considered as a possible candidate for the sepsis multi-therapy.

High blood pressure is often associated to diabetes. Diabetes type I and type II is characterized by the deficiency or the inefficiency, respectively, of insulin, the principal hormone that in physiological conditions promotes the influx and metabolic use of glucose into the cells. The reduction in the effective levels and/or action of circulating insulin induces a concomitant elevation of the counter-regulatory hormones, such as catecholamines, glucagons and growth hormone (8). The raised cathecolamines, in particular, renders the vascular smooth muscle cell more susceptible to the vasoconstriction, promoting the release of calcium from the intracellular stores to the cytoplasm. Augmented intracytoplasmic calcium concentration increases the kinases activity and therefore the phosphorilation of myosin, leading to the vasoconstriction and in the end to hypertension. So, as the blood pressure is a resultant of the cardiac output and the peripheral vascular resistance, the increase in the vasoconstriction and consequently in the vascular resistance leads to high blood pressure. High blood flow, and so the creation of hydrostatic forces within the blood vessel, creates a dragging frictional force, simply called ‘shear stress’. The shear stress appears to be a particular important haemodynamic force, as it stimulates the release of vasoactive substances,

worsening the above described vascular situation, and changing gene expression, cell metabolism, and cell morphology (9). The blood is directly in contact with the endothelium within the vessel, so the endothelial cells play a critical role for the changing in the haemodynamic forces. Several investigators have demonstrated that endothelial cells may actually be sensitive to the magnitude of the shear gradient (10, 11). The endothelium impairment promotes the release of adhesion molecules, like ICAM-1 or VCAM-1, of prothrombotic factors, growth factors and vasoactive substances.

The first important pattern to be involved in this process is the endothelial constitutive nitric oxide synthase (ecNOS). This enzyme promotes the rapid increase in nitric oxide (NO), which can either evoke anti-platelets aggregation or oppose to the synthesis of growth factors, that could increase the vessel wall thickness. The production of NO, the major vasodilator, could represent an opposite pathway to the vasoconstrictor agents, but the shear stress increases the amount of radical oxygen species (ROS) produced by the endothelium. ROS react with NO producing radical nitrogen species (RNS), that can oxidise the tetrahydropterin (BH4), fundamental cofactor for the eNOS activity (12). The resultant of these processes is the production of superoxide anion ($O_2^{-\cdot}$) and peroxynitrite ($ONOO^-$) (13), which lead to the deleterious oxidative stress.

The oxidative stress induces the modification of the physiological cellular structure of lipids, proteins and in particular of the DNA. The DNA structure alteration is quickly recognised by some ‘sentinel’ enzymes, like poly(ADP-ribose) polymerase-1 (PARP-1), which is implicated in the DNA repair and maintenance of genomic integrity (7). A relevant importance has the regulation by PARP-1 of the production

of inflammatory mediators via NF-kB, AP-1, Oct-1 activation. Among the mRNA protein transcripted, there are adhesion molecules and cytokines and chemokines, which initiate an inflammatory process. Thus, a metabolic syndrome like diabetes can turn into an inflamed *status* characterized by high vascular reactivity to vasoconstrictor agents.

Recently, a third biological gas was discovered, hydrogen sulphide (H_2S). It is produced by the conversion of L-cysteine by cystathionine β-synthase (CBS) or cystathionine γ-lyase (CSE). Actually, it has been shown that the final target of H_2S are the $K_{ATP.}$channels The opening of K_{ATP} channels allows an efflux of potassium, thus hyperpolarizing the plasma membrane and preventing the entry of calcium into the cell, leading to the vasodilatation. K_{ATP} channels are physiologically activated by decreases in the cellular ATP concentration and by increases in the cellular concentrations of hydrogen ions and lactate (14), a mechanism that links cellular metabolism with vascular tone and blood flow. Reduced ATP and increased protons and lactate is a typical metabolic situation in sepsis (15). The reduced afflux of glucose to the cells and the consequent reduction of ATP are also typical of diabetes, but, in contrast to sepsis, K_{ATP} are less sensitive as the predominant effect of catecholamines on the vascular cells, evoking a major vessel constriction.

In our experimental conditions, the administration of exogenous H_2S, in the form of sodium hydrogen sulphide (NaHS), revealed an increased vasodilatation on rat diabetic aortic rings and mesenteric vascular bed, compared to the control. In contrast, the administration of the metabolic precursor, L-cysteine, did not reveal the same results as NaHS, may be due to the lower activity of the enzyme responsible of its catalysis in the diabetic disease. The discover of a new endogenous gas could be a useful tool for future therapies for the diabetes, which

deleterious macrovascular complication is hypertension. The actual therapy for the diabetes is the replacement of insulin, but a future possibility could be the use of exogenous hydrogen sulphide donors which could diminish the secondary deleterious effect of hypertension in diabetic patients. Though, the application of hydrogen sulphide donors in the diabetic pathology could have many side effects as it was shown that NaHS can either induce proliferation or apoptosis in human vascular smooth muscle cells (16). So, H_2S may behave in the same ambiguous NO manner.

Instead, the actual sepsis therapy comprehends vasoconstrictors, who are not really successful, and glucocorticoids for their anti-inflammatory characteristics. A more promising possibility could be the activation of pathways like PARP-1 and Nrf-2, which could microscopically defend the physiological *status* of vascular and epithelial as well as endothelial cells, and rhEPO as coadjuvant to the already adopted therapy. Furthermore, TLRs modulators may be other efficient coadjuvants in the sepsis therapy as their activation achieves the possibility of defending the host from the pathogen invasion, counterbalancing the recruitment of pro and anti-inflammatory cells as well as anti-oxidant cytoplasmic molecules.

References

1. Galley HF, Davies MJ, Webster NR. Xanthine oxidase and free radical generation in patients with sepsis syndrome. *Crit Care Med*. 1996: 24(10): 1649-53

2. Victor VM, Rocha M, De la Fluente M. Immune cells: free radicals and antioxidants in sepis. *Intern Immunolpharmacol* 2004: 327-347

3. Immenshuhu S, .Schroeder H. Heme oxygenase-1 and cardiovascular diseases. *Histol Hystopathol*. 2006, 21(6): 679-85)

4. Ishii T, Itoh K, Takafashi S, Sato H, Yangawa T, Katoh Y, Bannai S, Yamamoto M. Transcription factor Nrf2 coordinately regulates a group of oxidative-inducible genes in macrophages. *J Biol Chem* 2000; 275(21): 16023-9

5. Murphy ME, Brayden JE. Nitric oxide hyperpolarizes rabitmesenteric arteries via ATP-sensitive potassium channels. J Physiol 1995; 486: 47-58)

6. Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, Cohen RA. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* 1994; 368: 850-3

7. Archer SL, Huang JMC, Hampl V, Nelson DP, Shultz PJ, Weir EK. Nitric oxide and cGMP cause vasorelaxation by activation of a charibdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc Natl Acad Sci USA* 1994, 91: 7583-7

8. English P and Williams G. Hyperglycaemic crises and lactic acidosis in diabetes mellitus. *Postgrad Med J* 2004, 80: 253-261

9. Davies PF. Flow-mediated endothelial mechanotransduction. *Physiol Rev.* 1995;75:519–560

10. Davies PF, Remuzzi A, Gordon EJ, Dewey CF Jr, Gimbrone MA Jr. Turbulent fluid shear stress induces vascular endothelial cell turnover in vitro. *Proc Natl Acad Sci U S A.* 1986;83:2114–2117

11. DePaola N, Gimbrone MA Jr, Davies PF, Dewey CF Jr. Vascular endothelium responds to fluid shear stress gradients. *Arterioscler Thromb.* 1992;12:1254–1257)

12. Andrew PJ, Mayer B. Enzymatic function of nitric oxide synthases. *Cardiovasc Res* 1999; 43: 521-31

13. Virag L and Szabo C. The therapeutic potential of poly(ADP-ribose) polymerase inhibitors. *Pharmacol Rev* 2002, 54: 375-429

14. Menissier-de Murcia J, Niedergang C, Trucco C, Ricoul M, Dutrillax B, Mark M, Oliver FJ, Masson M, Dierich A, LeMeur M, Wlatzinger C, Chambon P, and de Murcia G. Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells. *Proc Natl Acad Sci USA* 1997, 94: 7303-7307

15. Rossi S., Damico M, Capuano A, Romano M, Petronella P, Di Filippo C. Hyperglicemia in streptozotocin-induced diabetes leads to persistent inflammation and tissue damage following uveitis due to reduced levels of ciliary body heme oxygenase-1. *Mediators Inflamm*. 2006, 2006(4): 60285.

16. Keung EC, Li Q. Lactate activates ATP-sensitive potassium channels in guinea pig ventricular myocites. *J Clin Invest* 1991, 88: 1772-7

Acknowledgements

I am grateful to a number of people who supported me to carry out this work. First and foremost, my heartily profound thanks gratitude and appreciation are addressed to my PhD supervisor Prof. Raffaella Sorrentino for her encouragement, help and kind support. She believed in me since I was an undergraduate student so that she invested in my scientific growth. She taught me how to be critical and able to read through scientific results. Her leadership, support, attention to detail, hard work, and scholarship have set an example I hope to match some day.

I also greatly thank Dr. Roberta d'Emmanuele di Villa Bianca for her invaluable professional and personal advice. Her suggestions, discussions and guidance were a real support to complete my PhD training.

An affective memory is related to my experience in Prof. Jane Mitchell's group at the Imperial College, London. She friendly and humanly welcome me in her group and immediately treated me as one of her students. She gave me the possibility of enlarging my scientific techniques knowledge. My experience in her laboratory was not only a technical learning but also a scientific mind growth, as well as a life experience.

I also want to thank Prof. Giuseppe Cirino. He was not my supervisor but I will always remember when he used to come to the laboratory and discuss all together about the in-progress scientific projects. It was his way to further stimulate and increase the interest in what we were studying. He has always been a willing and helpful person.

Another professional figure to thank is Prof. Aldo Pinto, with whom my group collaborated on some projects. I always admired his capability, his spread knowledge and confidence on the studied projects.

It is then a great pleasure to thank Annarita Di Lorenzo and Vincenzo Brancaleone, fellows PhD students, and Dr. MariaRosaria Bucci and Dr. Fiorentina Roviezzo for their friendship and moral support.

Then, I would like to thank Prof. Enrico Abignente, PhD Coordinator, who has always been helpful in every kind of question and problem proposed over the PhD three years training.

Completing a PhD is truly a marathon event, and I would not have been able to complete this journey without the aid and support of countless people who have participated in a subjective manner to my scientific and personal growth over the past three years of my PhD.