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"Role and function of the Ca^{2+}-dependent protease calpain-1 associated to membrane lipid rafts"

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ABSTRACT

The Ca^{2+}-dependent protease calpain-1 has been found constitutively present in lipid raft/caveolin-1 membrane microdomains isolated from endothelial bEnd5 cells. In this membrane localization calpain-1 is associated with some of its substrates, such as endothelial Nitric Oxide Synthase (eNOS) and the chaperone Heat Shock Protein 90 (HSP90), whereas the calpain-2 and the inhibitor calpastatin have not been detected.

Alteration in intracellular Ca^{2+} homeostasis, induced by either treatment with Ca^{2+}-ionophore A23187 or prolonged cell exposure to high glucose, leads to a significant decrease in the level of eNOS associated to lipid rafts, followed by a recruitment of HSP90 at this site. The decrease of eNOS is due not only to its Ca^{2+}-dependent release from the caveolin-1 microdomains, but also to its digestion mediated by calpain-1. The specific involvement of calpain-1 in digestion of eNOS is supported by the preventive effect of a synthetic calpain inhibitor (CI-2). However, the modifications observed in lipid rafts protein composition make bEnd5 cells more resistant against cell death caused by Ca^{2+} overload. In this context, calpain-1 seems to play a protective role against NO overproduction.

Alterations in eNOS, calpain-1 and HSP90 levels have been also detected in aorta of Zucker Diabetic Rats (ZDR). The digestion of HSP90 indicates an aberrant activation of calpain, due to a chronic alteration in Ca^{2+} homeostasis occurring in these animals, and thereby the transition from a physiological to a pathological cell condition. These changes cannot be detected in brain cortex, suggesting a tissue-specificity of the alteration in Ca^{2+} homeostasis related to diabetes.

A similar protein organization of lipid rafts has been also observed in neuroblastoma SK-N-BE cells, where calpain-1 is associated with NMDAR, HSP90 and neuronal NOS (nNOS), as well as in brain cortex of normotensive rats. Preliminary results have demonstrated that the

increase in $[Ca^{2+}]_i$, that characterizes the nervous tissue of hypertensive rats, leads to a protein reorganization of the lipid rafts.

The results presented in this thesis provide new information on a selective localization and role of calpain-1. In endothelial cells the protease and its associated substrates seem to have a crucial role in responding to fluctuations of $[Ca^{2+}]_i$. Further investigations will be necessary to analyse the role of the protease associated to lipid rafts in nervous tissue both in physiological and pathological conditions.

1. INTRODUCTION

1.1 Calpains

Calpains are a group of intracellular Ca^{2+}-dependent cysteine endopeptidases, which have been identified in almost all eukaryotes and bacteria, but not in archaebacteria. In humans, this group of enzymes includes 15 isoforms, some of which are present ubiquitously and other ones show a tissue-specific pattern of expression [1]. First identified in 1964, calpains take their name from *cal*modulin and pa*pain* that share some functional aspects and part of the amino acid sequence [2, 3]. Calpains are implicated in many pathological and physiological processes such as cytoskeletal remodelling, cell proliferation, migration, invasion, apoptosis and signal transduction [1, 2]. Following activation, calpains can catalyse the proteolytic processing of a large number of intracellular proteins leading to modification of cell behaviour and viability. Because the enzyme participates not only in normal intracellular signal transduction cascades, but also in various pathological states, the study of calpains has aroused great interest in wide areas of life sciences in both basic and clinical research in the last decades [3]. However, the physiological importance of calpains remains to be established. The involvement of calpains activity in various pathophysiological events has been reported, but usually as an aggravating factor. Although it is commonly accepted that the regulation of calpains is critical for maintenance of normal cell functions, uncertainties remain regarding when and how these enzymes undergo activation [2]. Genetic techniques have demonstrated the cause-effect relationships between calpain deficiency and defective tissue functions. These conditions, named "calpainopathies" are often incompatible with cell survival, demonstrating the physiological importance of calpains [2]. It is now clear that calpains are endowed with functional properties different from those that characterise other main intracellular proteolytic components such as proteasomes, caspases and autophagy. First of all, the digestion of calpains substrates, which generally occurs in a limited and selective manner, is considered a "proteolytic processing" since

the target protein does not undergo extensive degradation but often acquires novel functional properties. Furthermore, calpains do not require any protein modification or tag to recognize and hydrolyse the peptide bonds present in the consensus sequences of its substrates themselves are involved in substrates recognition [2].

The conventional members in the superfamily of calpains are heterodimers with different 80 kDa catalytic large L-subunits and 30 kDa regulatory small S-subunits. In humans, 15 genes code for the L-subunits (CAPN1, CAPN2, CAPN3, CAPN5, …, CAPN16) and 2 genes code for S-subunits (CAPNS1, also called CAPN4, and CAPNS2) [2]. The gene products of CAPN1/CAPNS1 and CAPN2/CAPNS1 are named calpain-1 and calpain-2, respectively. These heterodimers share the same 30 kDa regulatory subunit, but each one has a different 80 kDa catalytic subunit. These two calpains, which are the best characterized forms, are ubiquitous (only erythrocytes lack calpain-2) and are distinguished by the calcium requirement to undergo *in vitro* activation. In fact calpain-1 (also known as μ-calpain) needs 5 - 20 μM Ca^{2+}, while calpain-2 (also known as m-calpain) needs 400 – 800 μM Ca^{2+} [2]. CAPN1 and CAPN2 contain four domains/regions: the N-terminal anchor helix region, the CysPc Protease Core domain (PC1 and PC2), the C2 domain-like domain (C2L), and the penta-EF-hand domains (PEF(L)). CAPNS1 contains an N-terminal Gly-rich (GR) domain and a penta-EF-hand domain (PEF(S)) [2]. The domains structure of the catalytic subunits of the conventional calpains, CAPN1 or CAPN2, is defined as “classical” and includes the C2L, the PEF and the CysPc domain. Alternatively, “non-classical” calpains exclude C2L and/or PEF domains [2]. Nine out of 15 calpain genes encode the “classical” or “conventional” calpains, CAPN1, CAPN2, CAPN3, CAPN8, CAPN9, CAPN11, CAPN 12, CAPAN 13 and CAPN14, whereas CAPN5, CAPN6, CAPN7, CAPN10, CAPN15 and CAPN16 are defined as “non-classical” calpains [2]. The ubiquitous calpains, CAPN1, CAPN2 (except in erythrocytes), CAPN5, CAPN7, CAPN10, CAPN13, CAPN14, CAPN15, CAPN16, CAPNS1 and CAPNS2, play an essential role in all cells, in fact, defects in these isoforms may be lethal, as seen in $Capn2^{-/-}$ or $Capns1^{-/-}$ knockout

mice [2, 4]; whereas the tissue-specific calpains, CAPN3, CAPN6, CAPN8, CAPN9, CAPN11 and CAPN12 are involved in more specific cellular functions and their defects may cause tissue-specific phenotypes, such as muscular dystrophy caused by mutations in CAPN3 [2, 5].

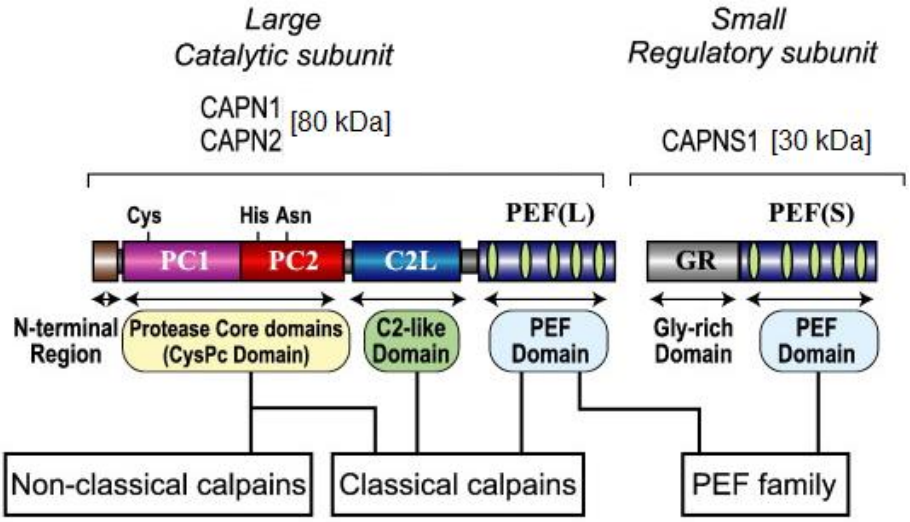

Figure 1: Schematic structure of calpain. Conventional calpains are composed of large catalytic and small regulatory subunits. Adapted from [2].

The catalytic mechanism of calpain is common to other cysteine-proteases, serine-proteases and threonine-proteases and depends on three amino acids collectively named "catalytic triads" [6]. In calpain the residue used as a nucleophile is the oxidized sulfur of the cysteine. In addition, the basic and acid residues needed for catalysis are provided by an histidine and an aspartate, respectively [7].

The physiological role of calpains is related to extracellular signals transduction mediated by changes in the permeability of the plasma membranes to Ca^{2+} or by the mobilization of this ion from intracellular stores. Indeed, the structural transitions of calpain during its activation process are triggered by Ca^{2+} addition [8]. The crystallographic analysis of the enzyme structure and the studies of the kinetics of Ca^{2+} binding have demonstrated that the onset of proteolytic activity appears only after a delay. This delay has been attributed to a Ca^{2+}-induced

conformational change making the active site of the enzyme accessible to the substrate. Furthermore, it has been demonstrated that Ca^{2+} causes the dissociation of the heterodimeric native calpain into its constitutive L- and S-subunits, with increased Ca^{2+} affinity than the native form, thereby yielding the active form of the protease [8, 9]. In the absence of Ca^{2+} the amino acid residues of the active site, which are crucial for the proteolytic activity, are not correctly positioned with respect to the substrate binding site, suggesting the requirement for a conformational change leading to the active form. Several studies have reported that the binding of Ca^{2+} causes an electrostatic switch, inducing a structural rearrangement of the active site leading to a correct positioning of the catalytic triad [8]. Indeed, the primary event in the activation process corresponds to the binding of Ca^{2+} to eight interacting sites, four in each of the two subunits, localized in calmoduline-like regions. Progressive binding of Ca^{2+} ions is linearly correlated with the dissociation of the heterodimeric enzyme, which reaches completion when all the binding sites are occupied [9].

Exposure to Ca^{2+} also induces calpain autoproteolysis. The 80 kDa calpain subunit, in presence of Ca^{2+}, undergoes intramolecular autoproteolysis that, by the removal of a peptide from the N-terminus, generates a 75 kDa species with an increased affinity for Ca^{2+}. Thus, such autoproteolytic event contributes to make the active site accessible to the substrate [9, 10]. An important question in the field of calpain research concerns the remarkable inconsistency of Ca^{2+} requirement necessary to activate the enzyme *in vitro* and the intracellular availability of this metal ion. In fact, although μM concentrations of Ca^{2+} are sufficient to activate purified calpain-1, the physiological amounts of cytosolic Ca^{2+} are in the range of 50 – 300 nM [11]. Since *in vitro* experiments have demonstrated that phospholipids, the most abundant type of lipid constituents in the cell membranes, lowered the Ca^{2+} requirement necessary to activate the protease, it has been proposed that microdomains close to the plasma membrane could represent favourable locations for the calpain activation. It has been also proposed that a limited amount of calpain, whose localization coincides with the spots where cell Ca^{2+}-concentration substantially

increases, is sufficient to maintain its physiological function [1]. The isolated 80 kDa catalytic subunit of calpain shows a 2-fold increased affinity for Ca^{2+} than the native heterodimeric form. However, only the autoproteolized 75 kDa form of the catalytic subunit shows a 20-fold increased affinity for Ca^{2+}, reaching a K_d closer to the physiological Ca^{2+} concentration [9].

An extensive literature suggests that calpains participate in a variety of cellular functions such as remodelling of cytoskeletal/membrane attachments during cell fusion and motility, processing of proteins involved in the control of the cell cycle, gene expression and apoptotic pathways [11]. However, the physiological functions of the calpain system and its regulation in living cells is not still completely defined. Moreover, the identification of some calpain isoforms lacking the amino acid residues necessary for catalysis suggests that in some circumstances calpains may play functions alternative to the proteolytic activity [11]. Aim of this thesis is to add information about the mechanism responsible of the intracellular regulation of the calpain proteolytic system. Many investigations have attempted to define the physiological functions of calpain using several different protease inhibitors in order to identify the cell functions affected. However, most of the inhibitors used are not absolutely specific for the calpains and effect the functions of other enzymes or have aspecific secondary effects. Consequently, the results of experiments based on the use of calpain inhibitors should be interpreted cautiously [11]. One of the best approaches available for studying the physiological functions of calpains is knocking out calpains genes. This allowed, for example, to determine that the 30 kDa regulatory subunit (Capn4) is crucial for animal vitality, since its lack causes a lethal phenotype in the mouse embryo. On the other hand, calpain-1 deletion only interferes with the platelets functioning and the coagulative process, but is not essential for mouse survival. Although it is commonly accepted that calpain-1 is involved in degradation of filamin, talin, and spectrin during platelet activation, the cleavage patterns and kinetics of degradation of both talin and filamin are normal in platelets from the $Capn1^{-/-}$ mice [11]. This finding supports the hypothesis that calpain-2, although present in very low amounts in mouse platelets, can digest the same substrates,

compensating for the absence of calpain-1. Instead, the role of calpain-2 in mouse embryonic development is absolutely crucial, because the deletion of Capn2 leads to a lethal phenotype already at a very early stage of gestation, suggesting that its essential functions cannot be compensated by calpain-1 [11].

Several studies have demonstrated that calpains are responsible for degradation of various cytoskeletal proteins implicated in cell division, cell motility or platelets activation. Implication of calpains in cytoskeletal remodelling is also related to their role in cytoskeletal/membrane interactions. For example, the β-integrin family of integrins is substrate of calpains. Integrin clustering or binding of ligands to specific receptors leads to calpain activation. Calpain mediated cleavage of both integrins and cytoskeletal proteins in focal contacts interferes with the interactions among these proteins resulting in disassembly of focal adhesions, cell rounding, loss of actin filament networks, increased rates of cell motility, and, in platelets, retraction of fibrin clots [11]. Calpains also participate in various signal transduction pathways through the cleavage of many phosphatases and kinases, such as PKC which becomes constitutively active after a limited proteolytic event catalysed by calpain. Moreover, calpains also participate in cell cycle regulation, through digestion of ciclin D1, that prevents mitosis and digestion of the tumor suppressor p53. Indeed, using synthetic calpain inhibitors it is possible to arrest the cell proliferation at the G1 phase with a substantial increase of the level of p53 [11]. The involvement of calpains in the regulation of gene expression has not been fully clarified. In fact, calpain is able to degrade several transcription factors, such as c-Jun, c-Fos or the aforementioned p53. However, if the calpains are effectively involved in digestion of transcription factors, this event occurs likely in the cytosol, because immunolocalization studies indicate that calpains are not generally located within the nucleus [11]. The participation of calpains to apoptosis seems limited to specific cell types, but these studies have been based on the use of synthetic inhibitors which often are not specifically directed towards calpain cathepsin, and proteasome. The use of combinations of proteasome inhibitors, cathepsin

inhibitors, and caspase inhibitors has not yet defined the precise role of calpains in this process. The understanding of the role of calpains in apoptosis is further complicated by the apparent ability of the calpains to cleave the caspases themselves as well as several proteins that regulate the progression of apoptotic steps. In some conditions, calpains may operate as a negative modulator of apoptosis through inactivation of caspases, whereas in other instances, they may work as positive regulators. This ambiguity suggests that the role of the calpains in apoptosis is highly cell and pro-apoptotic signal dependent [11].

In the last years, the putative involvement of calpains in a broad range of diseases has been the subject of many studies. Although calpains play a role in several pathological conditions such as neurodegenerations, ophthalmic diseases, cancer, myopathies, ischemic diseases, diabetes and endothelial dysfunction [1, 11, 12], only in a few cases mutations in the calpains genes cause clear pathological states, such as calpain-3 related muscular dystrophy, tumorigenesis associated to downregulation of calpain-9, or type 2 diabetes related to calpain-10. In most of these calpain-associated pathologies, the calpains do not represent the genetic cause. Instead, loss of Ca^{2+} homeostasis in cell can lead to a deregulation of calpain activity, accompanied by degradation of calpain substrates. Although it has been shown that calpain inhibitors can prevent or delay the onset of calpain-associated pathologies, these studies have been usually based on the use of synthetic inhibitors that are not completely specific for calpains and often cannot be used *in vivo* [11]. The increase in intracellular Ca^{2+} concentration that occurs in these pathological conditions is generally not sufficient to induce proteolytic activity of calpains, especially of the high Ca^{2+} requiring calpain-2. In some instances, Ca^{2+} may reach levels that could activate calpain-1 but, rather than activating calpains directly, it seems more likely that the alteration of Ca^{2+} homeostasis affects the regulation of calpain activity [11].

Different cells and different signalling pathways involve either calpain-1 or -2 but not both, and the two isoforms can play different roles in the same cell depending on the signal that the cell receives [11]. Calpain-1 and calpain-2 have been detected in almost every vertebrate cell, but

different tissues/cells differ widely in their ratios. For example, human platelets and erythrocytes have no detectable calpain-2, whereas bovine platelets and vascular smooth muscle have no detectable calpain-1 and skeletal muscle contains comparable amounts of the two isozymes [11]. It is possible that calpain-1 and -2 can perform similar physiological functions but in response to different cellular stimuli, also because of their different Ca^{2+} requirement. In fact, they have analogous specific cleavage sites, although often on different substrates. Previous studies revealed that calpain-1 and -2 are predominantly distributed in the cytosol, with a modest predominance around the nuclear region in cell resting conditions. Immunolocalization studies showed that in human erythrocytes 93% of the calpain is cytosolic and the remaining 7% is associated with the plasma membrane. In response to Ca^{2+} signaling, calpains translocate to the plasma membrane, identified as a cell site suitable for their activation [11, 13]. Several studies have suggested that a considerable fraction of calpains are not free in the cytosol, but rather associated with subcellular structures. In the skeletal muscle these structures are myofibrils, whereas in other cells they may be cytoskeletal filaments [11]. It has been estimated that, in a skeletal muscle cell, the concomitant activation of all calpain molecules could destroy some components of sarcomere, such as the Z-disks in less than 5 minutes. Consequently, in living cells calpain must be finely regulated not only by its Ca^{2+} requirement, but also by changes in its intracellular location, accessibility to substrates, and also by phosphorylation as shown for calpain-2 inactivation by PKA mediated phosphorylation [14]. In addition, it has been clearly established that calpain activity is also regulated by its natural protein inhibitor, calpastatin [11].

1.2 Calpastatins

Calpastatin, that does not share any sequence homology to any other protein, has been identified as the unique endogenous inhibitor specific for conventional calpains [15]. It is an approximately 120 kDa heat-stable protein resistant to a broad variety of denaturing agents and

ubiquitous in mammalian tissues. Different promoters or alternative splicing mechanisms allow each cell to produce different calpastatin polypeptides from a single gene. How many different start sites of transcription or translation might be used in different tissues or under different physiological states remain to be elucidated [11]. At present four main types of calpastatin with different N-terminal sequences and other four isoforms with internal deletions generated by alternative splicing have been identified. The various calpastatin forms show different inhibition efficiency. However, the physiological significance of the at least eight calpastatin isoforms is still unclear. [11, 16].

The full-length calpastatin contains a N-terminal XL-domain, followed by a L-domain and four repetitive inhibitory domains (I-IV domains) [16]. Three highly conserved regions, termed A, B and C, are present in the four repetitive inhibitory domains. Each of these conserved regions is able to interact preferentially with different calpain domains. Particularly, region B, interacting with the active site of calpain, seems to be the inhibitory centre of calpastatin, whereas regions A and C potentiate the inhibitory activity [2, 15, 16]. Each inhibitory domain can inhibit one calpain molecule, hence, potentially, calpastatin can simultaneously bind and inhibit four calpain molecules. Both the XL- and L-domain lack inhibitory activity. Their function is not yet completely clear, but it has been proposed that they can play a regulatory role on calpain-calpastatin interaction [17].

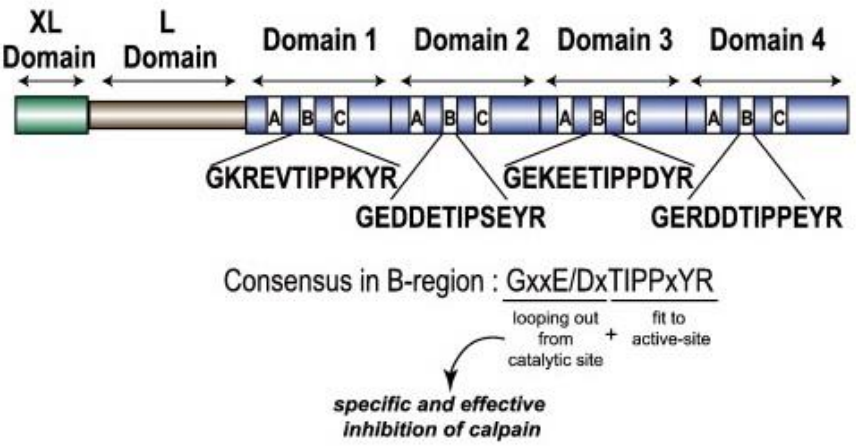

Figure 2: Schematic structure of the full-length calpastatin. The conserved 12-amino acid sequence in B-region (GxxE/DxTIPPxYR) is essential for inhibitory activity. Adapted from [2].

The interaction between calpain and calpastatin can occur in the absence of Ca^{2+} or at very low Ca^{2+} concentrations, reflecting the in *vivo* conditions and depending on the molecular structure of the calpastatin isoform involved. The L-domain seems to play a regulatory role on calpain activation [18]. A Ca^{2+}-independent association of the calpain-calpastatin complex can occur through a binding sequence localized in the L-domain of calpastatin. Conversely, the absence of L-domain makes this protein-protein interaction strictly Ca^{2+}-dependent [19]. Because the concentration of Ca^{2+} required for calpain-calpastatin association is lower than that required by calpains to initiate their proteolytic activity, and because immunolocalization studies suggest that calpains and calpastatin are frequently co-localized in cells, somehow cells must be able to allow calpain activation, even in the presence of its inhibitor [11]. Some of the mechanisms proposed to obtain this result involve the translocation of the calpain away from calpastatin or the reduction of Ca^{2+} requirement for calpain activation without affecting Ca^{2+} requirement for calpastatin binding. Furthermore, phosphorylation of calpastatin has been also reported as a post-translational mechanism to control the functional role of calpastatin. Hence, it is possible to hypothesize that different combinations of calpastatin phosphorylation/calpain

phosphorylation might completely prevent calpastatin binding and calpain inhibition at concentrations of Ca^{2+}sufficient for calpain activation [11, 15].

In addition to its role as calpain inhibitor, calpastatin is also a potential substrate of the enzymes. The calpains mediated limited digestion of native calpastatin, can result in the release of four still active inhibitory units that, by amplifying the cell inhibitory capacity, can play a crucial role in controlling calpain activation in cells with an altered Ca^{2+} homeostasis [17]. Indeed, the four free repetitive inhibitory domains of calpastatin are potentially available to counteract aberrant calpain activity [17]. Hence, this conservative process could represent a defence mechanism against the onset of an uncontrolled degradation of intracellular calpain substrates. The proteolysis of calpastatin has been mostly detected under conditions of excessive or prolonged $[Ca^{2+}]_i$ dysregulation, such as in neurodegenerative diseases [17]. However, in pathologies related with Ca^{2+} alteration, overexpression of calpastatin inhibitory units successfully prevents the excessive digestion of calpain targets [19, 20].

Under resting conditions, calpastatin appears homogenously distributed in cytosol, with a discrete preponderance around the nuclear region, but also in nucleus or in nuclear invaginations [13]. Hood *et al* have reported that calpastatin and the two ubiquitous calpain isoforms are associated with subcellular organelles, such as endoplasmic reticulum and Golgi apparatus [21]. After a stimulation that increases the intracellular Ca^{2+} concentration, calpastatin, unlike calpain, does not translocate to plasma membrane [13]. This dissociation of calpain from its inhibitor allows the protease activation and the cleavage of its substrates [21].

1.3 Interaction between calpain-1 and a few of its substrates

To fulfil its physiological functions calpain requires specific recognition of digestible substrates, selective cellular localization and proper mechanisms for regulating its activation and activity. It has been reported that an aliquot of calpain-1 is permanently associated to

multiprotein complexes in plasma membrane, where the protease is involved in the regulation and turnover of both enzymes and channels/receptors, such as the N-Methyl-D-Aspartate Receptor (NMDAR) [22, 23], one of the three types of ionotropic glutamate receptors, possessing an intrinsic ion channel. Its activation requires simultaneous binding of the two agonists glutamate and glycine along with membrane depolarization, but the receptor can be also activated by several endogenous and exogenous agonists [23]. NMDAR is implicated in various physiological functions in the nervous system, such as synaptic plasticity and memory function, but it is also involved in neurological diseases, such as epilepsy, ischemia, stroke and neurodegenerative disorders. [23]. NMDAR has been identified as a calpain target by several *in vitro* and *in vivo* studies. The receptor activation leads to entrance of Ca^{2+} into the cells followed by several down-stream intracellular events, including calpain activation. Over-stimulation of NMDAR causes an excessive influx of Ca^{2+}, which can lead to excitotoxicity [22, 23]. NMDAR is a heterotetrameric channel constituted of four subunits, usually two NR1 and two NR2 subunits. The NR1 subunit is not a calpain target, whereas the NR2 A-C subunits can be cleaved at the C-terminal region by calpain-1. The NR2 amino-terminal fragment produced by calpain-1 is still able to associate with NR1, forming a functional receptor [23]. However, Averna *et al* have reported that the digestion of NR2B subunit leads to its internalization into vesicles, together with NR1 [22]. While in resting conditions small amounts of NMDAR are detectable in intracellular membranes, following NMDAR stimulation most of the receptor molecules are internalized [22]. By digesting NMDAR and other associated intracellular proteins, calpain-1 can regulate the physiology of glutamatergic transmission. Consequently, it is plausible that, in pathological conditions, such as neuronal glutamate excitotoxicity, the activated protease can decrease the cell Ca^{2+} influx through this channel by promoting the removal of NMDAR from the plasma membrane. Thus, calpain-1 mediated cleavage might also have neuroprotective effects against NMDAR over-activation [22, 23].

The chaperone Heat Shock Protein 90 (HSP90) has been identified as a protein able to control calpain-1 activity [24]. HSP90 is a homodimeric ATPase, which contributes to the correct maturation and folding of a large variety of newly synthesized polypeptides and of several denatured or misfolded client proteins [25]. HSP90, interacting with calpain-1, but not with calpain-2, forms a complex in which the protease retains its catalytic activity, but acquires a reduced affinity for Ca^{2+}. Thereby, HSP90 participates in in regulating the activation of the protease [22, 24]. This could represent an alternative intracellular mechanism for the control of the protease, that operates near the plasma membrane, a site where calpastatin is not detectable. In fact, it has been demonstrated that in resting human leukemic T cells (JA3), cytosolic calpain-1 is associated with HSP90, and the chaperone competes with calpastatin for association to the protease. Thus, two different complexes can be produced in the cell: the first one containing calpain-1 and calpastatin, in which both calpain proteolytic activity and translocation to the internal surface of the plasma membrane do not occur; the second one containing calpain-1 and HSP90, in which calpain partially retains its activity and its trafficking is not impaired [24]. The formation of the complex between HSP90 and calpain-1 can prevent the total inhibition of calpain-1, leaving the protease free from calpastatin restriction. This occurrence could explain how calpain-1 can translocate and express proteolytic activity *in vivo*, although the amount of calpastatin largely exceeds that of calpain [24]. HSP90 is also inserted in the multiprotein NMDAR cluster, where the chaperone seems to control the physiological amount and activity of the resident calpain-1. However, HSP90 is also a calpain-1 substrate and a prolonged or intense calpain activation leads to the degradation of the chaperone [26]. The specific assembly of the calpain substrates with other regulatory proteins in membrane clusters could represent a valid model to explain how calpain can reach these structures, catalysing a selective, limited and controlled proteolysis [24].

Moreover, several reports have indicated that HSP90 and calpain-1 also participate in the Nitric Oxide Synthase (NOS) activation pathway [26-28]. NOSs are a family of Ca^{2+}-dependent

enzymes which catalyse the production of Nitric Oxide (NO) from L-arginine and oxygen. Three distinct isoforms have been identified in mammals: neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2) and endothelial (eNOS or NOS-3) [29, 30]. NO is an essential cellular signalling molecule, which has multiple paracrine functions, depending on the cell type which synthetize NO, the NOS isoform involved and the causal chain of events responsible of the NO production [30]. The neuronal isoform of NOS is a 155 kDa protein distributed both in the cytosol and associated with the plasma membrane. nNOS is constitutively expressed in nervous tissues, where it synthetizes NO in response to glutamate. nNOS is involved in synaptic plasticity, smooth muscle relaxation, central regulation of blood pressure and it also plays a role in cell communication [30, 31]. This enzyme is also involved in hypoxic or ischemic processes, playing either a neurotoxic or a neuroprotective role. Indeed, NO over-production may be neurotoxic, leading to the production of Reactive Oxygen Species (ROS), excitotoxicity, inflammation, apoptosis and brain injury [31]. The endothelial NOS is a 135 kDa protein constitutively expressed by vascular endothelial cells and it is predominantly associated to the plasma membrane. eNOS is mainly involved in vasodilation and it is activated in response to acetylcholine [30, 31]. eNOS can play both a vasoprotective and a neuroprotective role in ischemic processes, contributing to maintain a proper blood flow and preventing vascular injury. Diminished NO production has been implicated in pathogenesis of hypertension and in vascular disorders, such as atherosclerosis [31, 32]. The inducible NOS is a 130 kDa enzyme and, unlike the other two isoforms, is Ca^{2+}-independent. Following its identification in rat macrophages, predominantly distributed in the cytosol, it was successively detected in other cell types, such as human neutrophils, chondrocytes and hepatocytes. iNOS seems to be mainly implicated in cytotoxic actions of immune cells and inflammation and its expression and activation are induced by cytokines [29, 30].

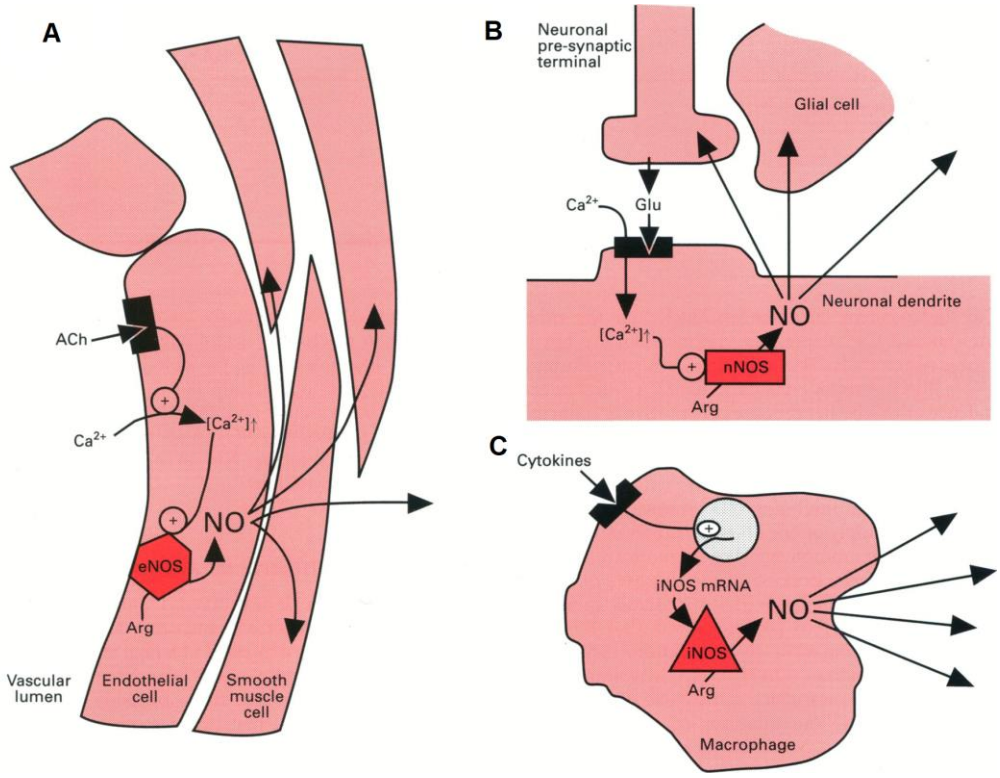

Figure 3: NO synthesis by the three NOS isozymes. (A) synthesis of NO by eNOS in endothelial cell stimulated by acetylcholine (ACh); (B) synthesis of NO by nNOS in a neuronal dendrite stimulated by glutamate (Glu); (C) expression of iNOS and synthesis of NO in a macrophage stimulated by cytokines. Adapted from [30].

It has been demonstrated that both the endothelial and the neuronal isoforms of NOS are calpain substrates [26, 27]. The limited digestion of nNOS is a conservative process without appreciable loss of catalytic activity of the synthase, whereas the degradation of eNOS occurs with a concomitant loss of protein and catalytic activity [27]. Also in this case HSP90 seems to play a crucial role in regulating the digestion of the two isozymes mediated by calpain. In basal conditions, HSP90 and NOS are associated in a heterocomplex in which the two substrates are almost completely resistant to calpain digestion, probably due to a structural steric hindrance [27]. Therefore the extent of NOS degradation depends on the level of HSP90 expression [27, 28]. eNOS is much more vulnerable to calpain mediated digestion than nNOS. Indeed, HSP90 is more abundant in nervous tissue than in the endothelial tissue Particularly, in rat brain, which

contains a large excess of HSP90, nNOS is almost completely associated with this chaperone. Thus, following calpain activation, nNOS undergoes a limited proteolytic cleavage, even if it maintains its catalytic activity [28]. Instead, in aorta, which contains lower levels of HSP90, eNOS cannot undergo a complete association with the chaperone. In this condition, eNOS degradation result in the loss of synthase protein and consequently of catalytic activity [28]. The different ratios of HSP90/NOS displayed in the two tissue types seem responsible for the conservative or non-conservative digestion of NOS isozymes [26, 27].

Specifically, in resting conditions the membrane-associated endothelial NOS is localized in caveolae, which are a specialized subset of lipid rafts widely present in the plasma membrane of endothelial cell. Interaction of eNOS with caveolae inhibits its synthase activity [32, 33]. eNOS can be directly activated by an increase in intracellular Ca^{2+}, as well as by phosphorylation mediated by upstream kinases [32]. Once activated, eNOS dissociates from the plasma membrane and translocates to the cytosol. This removal from caveolae is an essential step required for the activation of the synthase and NO production [26]. However, eNOS is a highly sensitive calpain-1 substrate and the elevation of $[Ca^{2+}]_i$ required for eNOS activation also leads to calpain-1 activation through its translocation to plasma membrane. Since eNOS degradation mediated by calpain is limited by the presence of HSP90 [26], the increase of Ca^{2+} concentration leads to the formation of a eNOS-HSP90 complex in which the synthase is protected from the proteolytic attack of activated calpain. On the other hand, cell treatment with a HSP90 inhibitor causes a massive eNOS degradation and suppression of NO production [26].

1.4 Membrane lipid rafts

The plasma membrane regions defined caveolae are invaginations of 50-100 nm and can be a subcategory of lipid rafts enriched in cholesterol, glycosphingolipids, sphingomyelin and lipid-anchored membrane proteins [32]. Caveolae are present in several cell types, but

particularly abundant in endothelial cells [32 - 35]. One of the functional roles played by these specialized membrane regions consists in the regulation of some Ca^{2+}-dependent signalling activities. In fact, several Ca^{2+}-dependent enzymes, such as eNOS and its associated proteins are resident in caveolae [32, 33]. In the last decades, many information on endothelial cell responses to external stimuli have been acquired through the identification of signalling molecules able to interact with caveolae and responsible of modifications of the caveolae protein profile. The physical structure of lipid rafts is quite unique with a highly ordered liquid state that differs from the surrounding disordered phospholipid bilayer. This peculiar characteristic is mainly due to the enrichment of specialized lipids, which determine a distinct density of these microdomains with respect to the rest of the plasma membrane, and makes them resistant to solubilisation by non-ionic detergents, such as Triton X-100 [35]. Such physical properties allow to isolate the membrane rafts from the rest of the cellular membranes, by applying several protocols, carried out using detergents or detergent free methods [35].

One of the major structural protein in caveolae is caveolin-1, a 21-24 kDa integral membrane protein. Caveolin serves as the structural component of caveolae, while also functions as scaffolding protein, capable of recruiting several signalling molecules, as well as regulating their activity [33, 34]. The direct association of eNOS and caveolin-1 seems to regulate the synthase activity. In particular, in resting conditions, ceveolin-1 keeps eNOS sequestered in caveolae, inhibiting its catalytic activity. Thus, the synthase activation required the dissociation of the enzyme from caveolin-1 [26, 33].

Membrane lipid rafts seems to function both as endocytotic/exocytotic carriers and as signal transduction organizing centres. In fact, they are involved in compartmentalization of several signal-transducing molecules in membrane microdomains [32, 33]. Moreover, experimental evidences have suggested the significant role played by lipid rafts in many biological processes, such as apoptosis, viral infections, cell adhesion and migration, synaptic transmission and organization of the cytoskeleton. Many of these physiological processes are

also known to require Ca^{2+} and thus it has been hypothesized that the lipid rafts can regulate these processes by modulation of Ca^{2+} signalling [35] and regulation of intracellular Ca^{2+} homeostasis. Specifically, the lipid rafts can regulate Ca^{2+} signalling by a direct activation of Ca^{2+} channels, by operating signal transducers or by regulation of the trafficking of key proteins involved [35]. The link between lipid rafts and Ca^{2+} signalling is supported by the fact that caveolae are also enriched in molecules that regulate Ca^{2+} influx across membrane, such as IP_3 receptor-like, Ca^{2+}-ATPase or members of the Transient Receptor Potential (TRP) family of capacitative Ca^{2+} entry (CCE) channels [35, 36]. Ca^{2+} is one of the simplest, yet perhaps the most versatile cellular messenger. It is the major signalling ion in both excitable and non-excitable cells, where it participates to a myriad of critical processes ranging from cell growth to cell death. Stimulation of cells by various endogenous agonists or pharmacological agents leads to either a Ca^{2+} influx across the plasma membrane or a release from intracellular stores. The latter phenomenon, in particular the release of Ca^{2+} from stores present in the Endoplasmic Reticulum (ER), can in turn activate a store-operated Ca^{2+} channel [35] that through Ca^{2+} influx from extracellular space also refill the ER stores. This mechanism, named Store-Operated Ca^{2+} Entry (SOCE), involves some members of TRPC and Orai channels [35, 36]. Several studies indicate that key proteins implicated in Ca^{2+} signalling are localized in discrete plasma membrane lipid rafts microdomains and that caveolae are potential sites of extracellular Ca^{2+} entry in response to ER Ca^{2+} depletion [35, 36]. In fact, many of the regulators and channels, such as SOCE channels, involved in Ca^{2+} signalling have been found in lipid rafts. Clustering of Ca^{2+} channels and their modulators in such membrane microdomains can provide a spatial segregation and regulation of Ca^{2+}-dependent events. Thus, membrane rafts represent a functional domain wherein as highly specific signalling events can be efficiently executed [35, 36].

In addition to Ca^{2+} channels and effectors of Ca^{2+} signalling, lipid rafts also compartmentalize many other signal transduction molecules and receptors which regulate

multiple cell functions [35, 37]. Epidermal Growth Factor (EGF) and Platelet-Derived Growth Factor receptors, muscarinic and α-, β-adrenergic receptors, serotonin and oxytocin receptors, MAP kinase, PKC, PLC and G proteins, among others, have been detected in lipid rafts microdomains [35, 37]. Since many signalling cascades and several physiological processes are associated to caveolae, it is conceivable that several diseases might be related to ablation or mutations of caveolin. Experiments performed in caveolin-deficient mice have demonstrated the functional significance of this protein in a variety of pathologies, including diabetes, cancer, cardiovascular diseases, pulmonary fibrosis and muscular dystrophies [34].

Since the plasma membranes are now considered dynamic interfaces also endowed of specialized microdomains favourable to the activity of membrane-associated proteins [38], both quantitative and qualitative variations in membrane lipid composition have been related to changes in the regulation of membrane protein functions. For example, Robinson *et al* have reported that the P2X7 purinergic receptor channel, associated with cholesterol-rich lipid rafts, is potentiated by depletion of cholesterol, suggesting that the influx through this nonselective cation channel can be regulated by changes in lipid rafts composition [39]. Furthermore, several alterations in brain lipid composition have been associated with both normal and pathological aging processes [38]. Overall these observations are consistent with the concepit that rearrangements in the composition of lipid rafts may play a crucial role in several functions played by these functional platform units of the cell membrane.

It has been shown that calpain-2 interacts with lipid rafts in human T-cell and cleaves its targets during Ag-specific T-cell signaling and immune synapse formation [40]. More recently active calpain-2 has been also identified in myotube caveolae where the protease processes vimentin, desmin and vinculin [41]. The possible localization and activation of calpain-1 at the lipid rafts has not yet been described.

2. AIM OF THE THESIS

The aim of this thesis was to evaluate the possible presence of calpain-1 in membrane lipid rafts, to analyse the interaction/association of the protease with a few of its substrates and study the relevant function. Since lipid rafts are related to Ca^{2+} signalling [32 – 36], it is conceivable an involvement of these microdomains in activation of the Ca^{2+}-dependent proteolytic system.

Previous studies have indicated that dysregulation of Ca^{2+} homeostasis causes, in addition to an aberrant activation of the Ca^{2+}-dependent proteolysis, an altered NO production [1, 11, 12]. These events occur in several pathologies, such as hypertension and diabetes and are related to endothelial dysfunction [11, 12].

The relationship among calpain-1, NOS and the chaperone HSP90 and their association with lipid rafts have been analysed both in physiological conditions and following Ca^{2+} stimulation, using a murine endothelial cell line. For this purpose, a protocol has been developed for the isolation of membrane lipid rafts, adapted from various papers in the literature. Then, it has been verified whether the lipid rafts microdomains undergo a protein reorganization following exposure to stimuli that increase the $[Ca^{2+}]_i$. Furthermore, through a cell viability assay it has been evaluated the role of calpain-1 and NOS in the endothelial cell. In support of the data obtained, some experiments have been carried out using animal models affected by Type 2 diabetes and by obesity and insulin resistance.

Furthermore, to establish if these membrane microdomains represent a specific site of activation and action of calpain-1 also in other cell types, the association of the protease with lipid rafts has been explored also in membranes isolated from a neuroblastoma cell line, where calpain-1 is associated to a NMDAR protein cluster. In order to obtain more information on the *in vivo* relationship between lipid rafts and alteration of Ca^{2+} homeostasis, an animal model characterized by a constitutive alteration of $[Ca^{2+}]_i$ has been utilised. Particularly, we have utilised tissues obtained from normotensive and genetically hypertensive rats, analysing the

protein rearrangement of the lipid rafts that occurs in the pathological condition. These data have contributed to enhance our knowledge about the localization, role and function of calpain-1 associated to these membrane domains, both in physiological conditions and in altered [Ca^{2+}] conditions.

3. MATERIALS AND METHODS

3.1 Reagents and antibodies

Tween® 20, Triton® X-100, calpain inhibitor-1 (CI-1), calpain inhibitor-2 (CI-2), Ca^{2+}-ionophore A23187, N_{ω}-Methyl-L-arginine acetate salt (L-NMMA), Neutral Red Solution (0.33%), mouse monoclonal anti-calpain-1 (calpain-1, subunit p80) clone 15C10, mouse monoclonal anti-calpain-2 (Domain III/IV) clone 107-82 and mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Sigma-Aldrich. ECL SELECT® Detection System and Protein G-Sepharose were obtained from GE-Healthcare. Rabbit polyclonal anti-caveolin-1, mouse monoclonal anti-neuronal NO synthase (nNOS), anti-endothelial NO synthase (eNOS), anti-HSP90 and anti-NR2B antibodies were purchased from BD Transduction Laboratories. Mouse monoclonal anti-NR1 was obtained from Millipore. Rabbit polyclonal anti-EGFR was purchased from Santa Cruz Biotechnology. Monoclonal anti-calpastatin (Domain IV) clone 1F7E3D10 was bought from Calbiochem. Protease Inhibitor Cocktail (100X), Horseradish Peroxidase (HRP)-linked anti-mouse and HRP-linked anti-rabbit secondary antibodies were purchased from Cell Signaling Technology.

3.2 Animals

For the experiments carried out in animals, 12-14 weeks old rats were used. For the first part of the study, rats (Charles River Laboratories, Wilmington, Massachusetts, USA) were divided into three groups: homozygote (fa/fa) male Zucker Diabetic Rats (ZDR), Zucker Fatty (obese) Rats (ZFR) and control heterozygote (fa/-) Zucker Lean Rats (ZLR). For the second part, rats (*Rattus norvegicus* Milan strain) were divided in 2 groups: normotensive and hypertensive rats. All the experimental procedures and animal care were conducted following the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the

Italian Ministry of Health in accordance with Decreto Ministeriale 116/1992 (protocol number 22698 of 17 September 2013). The related project dealt with the ethical and animal care aspects and was approved by the Committee set by the Ministry of Health at the National Institute of Health (Rome). Rats were housed in controlled conditions of temperature (22±1°C), relative humidity (50%), and lighting (12 h a day), and were fed by a standard rat chow containing 0.3% sodium and drunk tap water *ad libitum*. Rats were anesthetized with sodium pentobarbital (Sigma Chemicals) and sacrificed by decapitation. Brain cortex and aorta were rapidly removed, quickly frozen in liquid nitrogen, and stored at -80 °C.

3.3 Cell culture

Murine endothelial bEnd5 cells and human neuroblastoma SK-N-BE cells were maintained in continuous culture at 37°C (5% CO_2) with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 10 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine. All products used for cell culture were purchased from Euroclone S.p.A.

3.4 Isolation of membrane lipid rafts

For the isolation of membrane lipid rafts, a detergent-free method was developed and adapted from different protocols reported in Ref. [42 - 45]. All steps were carried out on ice. bEnd5 cells or SK-N-BE cells (35×10^6) were washed twice in phosphate-buffered saline (PBS), scraped from bottom of the flask and suspended in Tris 20 mM, pH 7.4, containing 250 mM sucrose (8.6% m/v), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 19 µM calpain inhibitor-1 and Protease Inhibitor Cocktail (lysis buffer).

Alternatively rat brain cortex was suspended in 5 vol (w/v) of lysis buffer, minced and homogenized in a Potter Elvehjem homogenizer. The cells or the homogenized tissue were then lysed by passage through 22 g × 3" needle 20 times, as described in [43, 45]. For the elimination of nuclei, lysates were centrifuged at 1000× g for 10 min and the resulting postnuclear supernatant was collected, whereas the pellet was re-suspended in 1 mL of lysis buffer and again lysed by passage through needle as above. After a second centrifugation at 1000 g for 10 min, the second postnuclear supernatant was pooled with the first one and the pellet (nuclei) was discarded. Protein quantification of the samples collected was carried out using the Lowry method as described in [46]. Samples containing 3 mg of protein in 2 mL were then adjusted to 45% sucrose by the addition of 2 mL of 90% sucrose in Tris 20 mM, pH 7.4, containing 0.15 M NaCl, and placed at the bottom of an ultracentrifuge tube. A discontinuous sucrose gradient was formed above by adding 4 mL of 30% sucrose and 4 mL of 5% sucrose as reported in [42]. Samples (total volume of 12 mL each) were subjected to ultracentrifugation for fractionation in a SW 28 rotor (Beckman) at 20,000 rpm for 18 h at 4°C in a SW28 rotor. This step allowed to separate different fractions depending on their density. From the top of each gradient, ten sequential fractions of 1.2 mL were collected, diluted in 3 mL of PBS and centrifuged at 100,000× g for 40 min at 4°C. The pellets were suspended in Laemmli loading buffer [47], heated for 5 min at 95 °C and submitted to sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) (15% for caveolin-1, 6% for the other proteins).

3.5 Immunoblotting (Western Blot)

For the immunoblot analysis of whole samples, aliquots of rat brain cortex and aorta were suspended in 5 vol (w/v) of ice-cold 50 mM sodium borate buffer, pH 7.5, containing 0.5 mM 2-mercaptoethanol, 2 mM ethylenediaminetetracetic acid (EDTA) and Protease inhibitor Cocktail,

minced, homogenized in a Potter Elvehjem homogenizer and lysed by 3 cycles of sonication. Protein quantification of crude extracts obtained from the tissues considered above was assayed by the method of Lowry and aliquots of crude extracts were suspended in Laemmli loading buffer, heated for 5 min at 95 °C and submitted to 6% SDS-PAGE.

Alternatively bEnd5 cells (3×10^6), differently treated as described in Results, were washed twice in PBS and lysed in 500 µL of ice-cold 50 mM sodium borate buffer, pH 7.5, containing 1 mM EDTA and Protease Inhibitor Cocktail by sonication. In order to separate the total membrane fraction from cytosolic fraction, following protein quantification, cellular crude extracts were centrifuged at 100,000× g for 10 min at 4 °C and the supernatant (cytosolic fraction) was collected separately from the pellet (membrane fraction). Aliquots (30 µg) of crude extracts, cytosolic and total membrane fractions were suspended in Laemmli loading buffer, heated for 5 min at 95 °C and submitted to SDS-PAGE (6%) and Western blot analysis. Fractions obtained from the isolation of membrane lipid rafts (as described above), after SDS-PAGE, were also submitted to immunoblotting.

Following electrophoresis, proteins were transferred to a nitrocellulose membrane (GE-Healthcare) by electroblotting as reported by Towbin *et al* [48]. To detect the various proteins analysed, the nitrocellulose membranes were probed with the specific primary antibodies listed in the section Reagents and antibodies. The immunoreactive signal was developed using the ECL SELECT® detection system, detected with a Bio-Rad Chemi Doc XRS apparatus, and quantified using the Quantity One 4.6.1 software (Bio-Rad).

3.6 Immunoprecipitation

The fraction containing the highest amount of membrane lipid rafts obtained as described above was solubilized in 500 µL of 50 mM sodium borate buffer, pH 7.5, containing 1 mM EDTA and Protease Inhibitor Cocktail + 0.1% Triton X-100 (immunoprecipitation buffer) and

incubated at 37 °C for 1 h. The soluble proteins was precleared with protein G-Sepharose for 20 min at room temperature (RT) and then incubated in the presence of the specific primary antibody at 4 °C, overnight. Protein G-Sepharose was then added to the sample and incubated for 1 h at RT. The immunocomplexes were washed three times with immunoprecipitation buffer without Protease Inhibitor Cocktail and Triton X-100, suspended in Laemmli loading buffer and submitted to 6% SDS-PAGE followed by Western blot, as described above.

3.7 Cell viability assay

Cell viability was measured using the Neutral Red Uptake assay, as reported by Repetto *et al* [49]. Briefly, the neutral red uptake assay provides a quantitative estimation of the viable cells in a culture. It is based on the ability of viable cells to incorporate the neutral red dye in the lysosomes. Cells were seeded in 96-wells plates and treated for the appropriate period. The plates were then incubated for 2 h at 37 °C with a medium containing neutral red. The cells are then washed and the dye was extracted in each well with a destaining solution. The absorbance was finally read using a spectrophotometer set at a wavelength of 540 nm [49].

3.8 Statistical analysis

Values are presented as means ± SEM. Significance of the differences was analysed by paired Student's t-test or ANOVA followed by post-hoc Tukey's test, using the Prism 5.0 GraphPad Software. The probability level taken to indicate significance was $p < 0.05$.

4. RESULTS

4.1 Calpain-1, eNOS and HSP90 are present in a protein complex associated to lipid rafts/caveolin-1 membrane microdomains

In the first part of this study, experiments were performed on bEnd5 cells, used as model of endothelial cell, in order to analyse the protein composition of lipid rafts/caveolin-1 membrane microdomains. First of all, by separation of total membranes fraction from cytosolic fraction, we verified the distribution of eNOS in bEnd5 cell. As previously reported [26, 32, 33], in resting conditions more than 95% of eNOS is associated with the membrane fraction (**Fig. 4**).

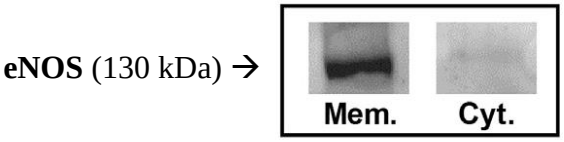

Figure 4: **Distribution of eNOS in bEnd5 cells**. Aliquots (30 µg) of cytosolic fraction (Cyt.) and total membranes (Mem.) from bEnd5 cells obtained as described in Methods were subjected to SDS-PAGE and immunoblot analysis was performed using anti-eNOS antibody. The protein is mostly present in the membranes fraction. The picture represents one of three different experiments performed.

bEnd5 cells lysate was fractionated on a discontinuous sucrose gradient by ultracentrifugation, in order to isolate lipid rafts microdomains, as described in Methods. Following fractionation, the protein concentrations of the fractions obtained were measured by the Lowry method (see **Fig 5 A**). For the identification of fractions containing lipid rafts, caveolin-1 was used as a marker, since it is particularly abundant in caveolae of endothelial cells [32 - 35]. After immunoblot analysis of the gradient fractions, most of eNOS was found in a single fraction (fraction 7), containing the marker caveolin-1, together with significant amounts of calpain-1 and HSP90. Both calpastatin and calpain-2 were not detectable in the various

fractions, suggesting a selective association of calpain-1 with these membrane regions, in which its natural inhibitor is absent (**Fig 5 B**).

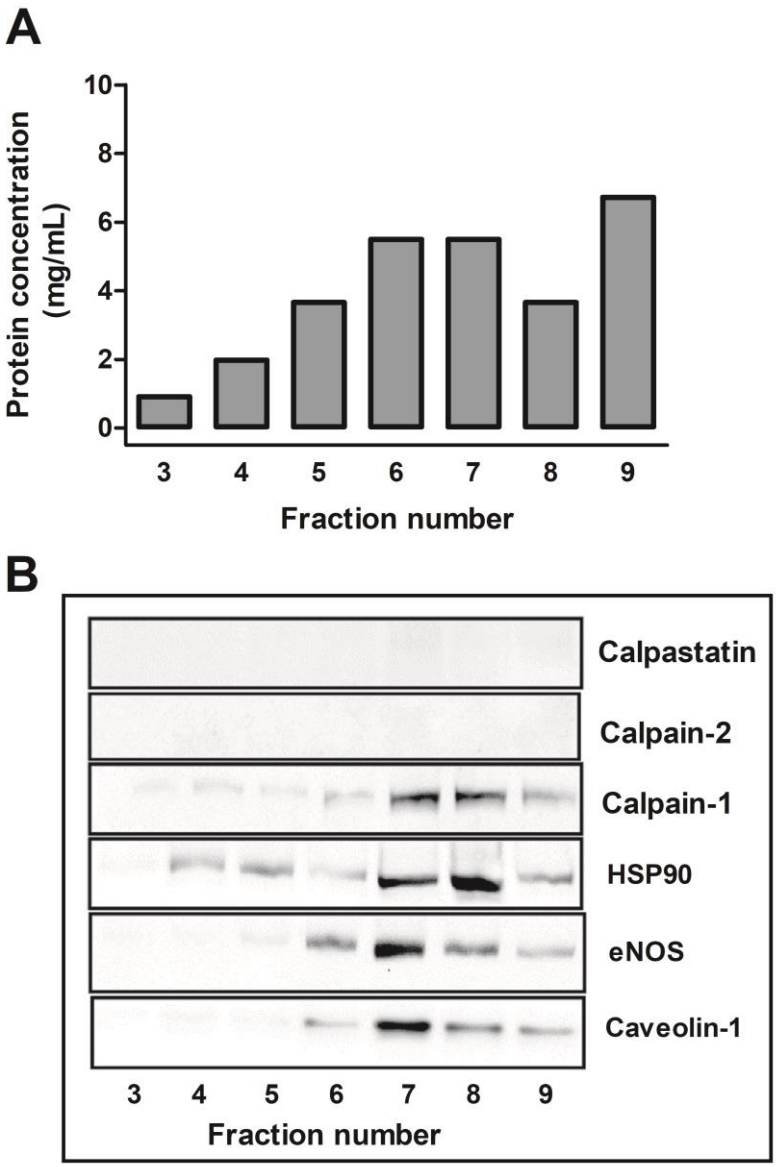

Figure 5: **Protein composition of lipid rafts/caveolin-1 membrane microdomains isolated from bEnd5 cells**. (**A**) bEnd5 cells (35×10^6) were lysed to obtain lipid rafts as described in Methods. The protein concentration of each fraction was measured and only the values referred to fractions 3-9, containing detectable protein amounts, are reported. (**B**) Aliquots (30 µg) of each fraction obtained in (**A)** were subjected to immunoblot analysis with the indicated antibodies. The pictures represent one of the three experiments performed. The lipid rafts distribution does not exactly reflect the trend in protein concentration among the fractions.

To verify if these proteins are physically associated, immunoprecipitation was performed on fraction 7, containing the most abundant amount of eNOS and caveolin-1. As shown in **Fig. 6,** using anti-eNOS antibody to perform the immunoprecipitation, we demonstrated that eNOS, calpain-1 and HSP90 are present in a single protein complex also associated with caveolin-1,.

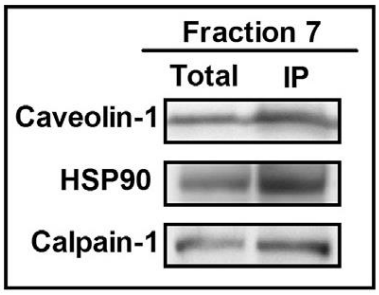

Figure 6: **Immunoprecipitation of eNOS associated to lipid rafts**. Aliquots (500 µg) of soluble proteins (Total) obtained from fraction 7 were immunoprecipitated using 2 µg of anti-eNOS antibody as described in Methods. 30 µg of Total and all the immunoprecipitated material (IP) were submitted to Western blot using the indicated antibodies. The picture represents one of three experiments performed.

4.2 Ca^{2+}-dependent protein modifications in caveolae

As already mentioned in Introduction, in endothelial cells the lipid rafts/caveolae represent platforms in the plasma membrane where Ca^{2+} signalling is locally regulated to induce specific cell responses [35]. It is conceivable that lipid rafts are involved also in regulating Ca^{2+} influx required for the activation of both eNOS and calpain-1 and that the chaperone HSP90 controls calpain-1 activation in these microdomains. Thus, the eNOS/calpain-1/HSP90 complex associated to caveolae could be used as a model to study the protein changes mediated by perturbations of Ca^{2+} homeostasis. As demonstrated by several reports [50 – 55], one of the conditions that induces alterations in $[Ca^{2+}]_i$ is the cell exposure to high glucose levels, as it occurs in diabetes mellitus. In endothelial cells, high glucose condition promotes dysregulation of Ca^{2+} homeostasis that leads to activation of Ca^{2+}-

dependent systems, such as the Ca^{2+}-dependent proteolysis [50 - 55]. In fact, it has been demonstrated that the endothelial dysfunction, a recurrent complication of diabetes, is also characterized by an aberrant activation of calpain [50, 53, 55].

Thus, in order to simulate a pathological condition, bEnd5 cells have been exposed to 25 mM glucose for 48 h (High Glucose, HG) (normally the glucose concentration in the cell culture medium is about 5 mM, which reflects the *in vivo* physiological concentration). Alternatively cells have been treated with 1 µM Ca^{2+}-ionophore (A23187) for 30 min, causing a significant increase in intracellular Ca^{2+} levels. Following treatments, lipid rafts/caveolin-1 membrane microdomains were isolated and the levels of eNOS, calpain-1 and HSP90 present in fraction 7, obtained from sucrose gradient as described above, were revealed by Western blot. As shown in **Fig. 7**, exposure to HG led to a 60% decrease in eNOS level as compared to control cells (C). The calpain-1 level was not changed significantly. Unexpectedly, the amount of HSP90 was approximately 2-fold increased.

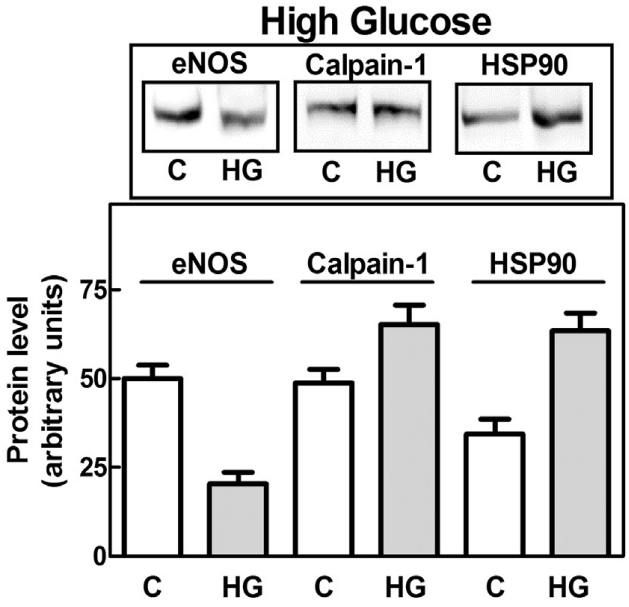

Figure 7: **Modifications of eNOS, calpain-1 and HSP90 levels in lipid raft/caveolin-1 membrane microdomains following HG treatment.** bEnd5 cells (35×10^6), cultured for 48 h in DMEM (C) or in DMEM + 25 mM glucose (HG), were lysed to isolate lipid rafts. Aliquots (30 µg) of gradient fraction 7 were subjected to immunoblotting with the indicated antibodies.. The immunoreactive material was detected and quantified. The reported values are means ± SEM of five independent experiments performed. The pictures represent one of the five experiments carried out.

Interestingly, as shown in **Fig. 8**, treatment with Ca^{2+}-ionophore led to very similar results. These data suggest that both events are directly associated with the increase in $[Ca^{2+}]_i$.

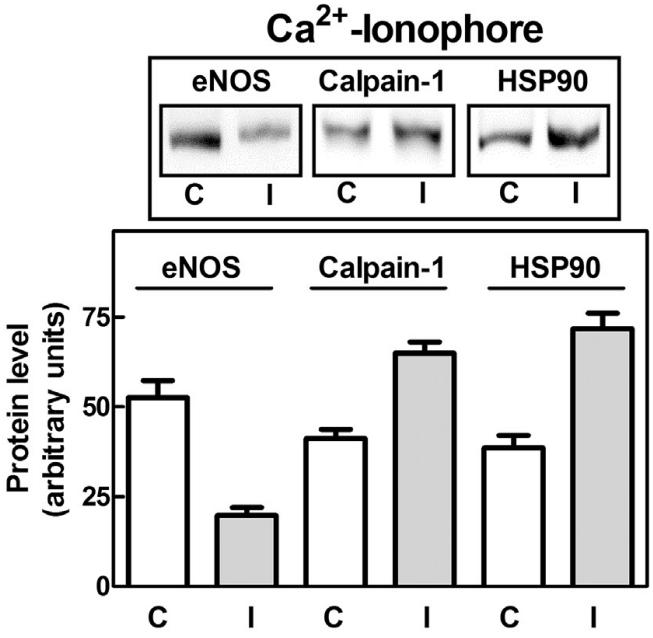

Figure 8: **Modifications of eNOS, calpain-1 and HSP90 levels in lipid raft/caveolin-1 membrane microdomains following Ca^{2+}-ionophore treatment.** bEnd5 cells ($35x10^6$), left untreated for 30 min in DMEM (C) or treated for 30 min with 1 µM Ca^{2+}-ionophore A23187 (I), were lysed to isolate lipid rafts. Aliquots (30 µg) of gradient fraction 7 were subjected to immunoblotting with the indicated antibodies.. The immunoreactive material was detected and quantified. The reported values are means ± SEM of five independent experiments performed. The pictures represent one of the five experiments carried out.

4.3 eNOS digestion is mediated by lipid rafts-associated calpain-1

The reduction in eNOS levels associated to lipid rafts/caveolin-1 membrane microdomains, following the treatment with either HG or Ca^{2+}-ionophore, could be explained through two concomitant events. As indicated by several reports [26, 27, 33] and already mentioned in Introduction, the Ca^{2+}-dependent activation of eNOS involves the dissociation of

the synthase from the caveolae and its translocation to cytosol. But, at the same time, under increased $[Ca^{2+}]_i$ conditions, the active calpain-1 digests its substrates, including eNOS.

To better characterize this process, bEnd5 cells have been treated with HG or Ca^{2+}-ionophore in the absence or in the presence of a cell permeable calpain inhibitor (CI-2) and total amount of eNOS was then evaluated. As shown in **Fig. 9 A** and **B**, the decrease in eNOS level, induced by HG or Ca^{2+}-ionophore treatments, was prevented by the presence of CI-2. Although CI-2 does not specifically inhibits calpains-1, it has previously been demonstrated that calpain-1 is involved in the degradation of eNOS, while calpain-2 regulates eNOS phosphorylation in endothelial cell [56]. Thus, we could confirm that eNOS digestion is mediated by calpain-1.

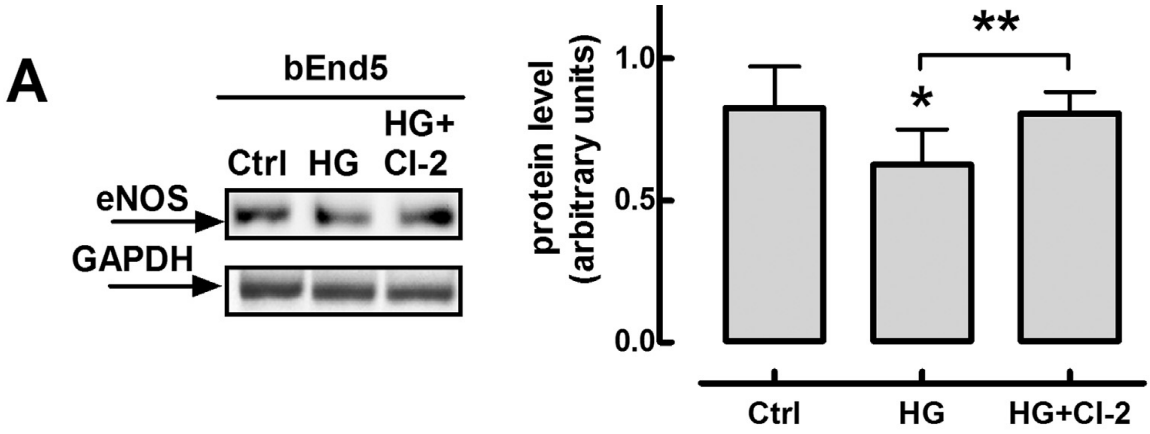

Figure 9 A: **Calpain-mediated digestion of eNOS following HG treatment.** bEnd5 cells (3×10^6) cultured for 48 h in DMEM (Ctrl), or in DMEM + 25 mM glucose (HG), or in DMEM + 25 mM glucose in the presence of 2 µM CI-2 (HG+CI-2) were lysed as described in Methods and aliquots (30 µg) of crude extracts were submitted to SDS-PAGE followed by immunoblotting and probed with the indicated antibodies. The immunoreactive bands were quantified. The reported values are means ± SEM of five independent experiments performed. eNOS quantification was normalized versus GAPDH immunoreactive band intensity, used as loading control protein. * significantly different as compared to controls (p = 0.0417); **p = 0.0204. The picture represents one of the five experiments carried out.

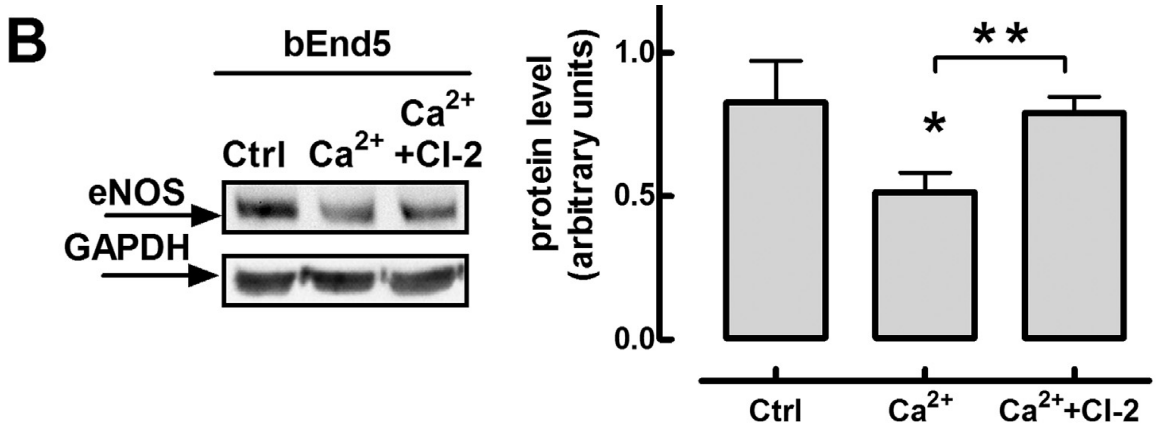

Figure 9 B: **Calpain-mediated digestion of eNOS following Ca^{2+}-ionophore treatment.** bEnd5 cells (3×10^6) left untreated for 30 min in DMEM (Ctrl), treated for 30 min with 1 µM Ca^{2+}-ionophore A23187 (Ca^{2+}) or incubated with 1 µM CI-2 for 30 min, followed by an incubation with 1 µM Ca^{2+}-ionophore A23187 for 30 min (Ca^{2+}+CI-2) were lysed as described in Methods and aliquots (30 µg) of crude extracts were submitted to SDS-PAGE followed by immunoblotting and probed with the indicated antibodies. The immunoreactive bands were quantified. The reported values are means ± SEM of five independent experiments performed. eNOS quantification was normalized versus GAPDH immunoreactive band intensity, used as loading control protein. * significantly different as compared to controls ($p = 0.0145$); **$p = 0.0403$. The pictures represent one of the five experiments carried out.

However, the decrease of eNOS observed in lipid rafts/caveolin-1 membrane microdomains, and reported in **Fig 7** and **Fig**. **8**, is more pronounced than that detected in total cell (**Fig. 9 A** and **B**). This discrepancy was explained by the fact that, following $[Ca^{2+}]_i$ increase, an aliquot of eNOS was dissociated from the plasma membrane as an active enzyme form and translocated to the cytosol. The amounts (expressed as %) of eNOS dissociated from lipid rafts or digested by calpain-1, following HG or Ca^{2+}-ionophore treatments, are reported in **Table 1**.

Cell treatment	eNOS distribution (%) [a]			
	Total [b]	Lipid rafts [c]	Cytosolic [d]	Digested [e]
None	100	100	-	-
HG	76 ± 5.7	40 ± 2.9	36 ± 1.8	24 ± 1.6
Ca^{2+}ionophore	60 ± 6.2	35 ± 2.5	25 ± 3.0	40 ± 3.7

Table 1: Digestion of eNOS and its release from lipid rafts.

All data are presented as the arithmetical mean ± SE.

(a) 100% is the level of eNOS measured in untreated cells.

(b) Values were extrapolated from Fig. 9 A and 9 B.

(c) Values were extrapolated from Fig. 7 and Fig. 8.

(d) Values were extrapolated subtracting the level of eNOS in lipid rafts (c) from that in total cells (b) measured following the same cell treatment.

(e) Values are the differences between the level of eNOS obtained from total untreated and treated cells.

In order to verify if calpain-1 resident in caveolae was the isoform responsible for this eNOS digestion, the gradient fraction containing lipid rafts/caveolin-1 microdomains (fraction 7), obtained from untreated bEnd5 cells, was incubated with a [Ca^{2+}] sufficient to fully activate lipid rafts-associated calpain-1 *in vitro*. As shown in **Fig. 10**, following the treatment, a 70% eNOS reduction was detected and, in this case, also HSP90 decreased, although at a lower extent (40 – 45%). These results demonstrated that resident calpain-1 is involved in eNOS digestion. The activation and expression of catalytic activity of resident calpain-1 is also promoted by the absence of its natural inhibitor calpastatin in caveolae (as shown in **Fig. 5**). As mentioned in Introduction and reported in [26], HSP90 is also a calpain-1 substrate and this explains the HSP90 reduction detected in isolated lipid rafts following exposure to Ca^{2+}. However, HSP90 is

also involved in regulating calpain-1 activity increasing the Ca^{2+} request for the protease activation [24]. Therefore, increase of lipid rafts-associated HSP90 observed in **Fig. 7** and **Fig. 8**, following treatment with HG or Ca^{2+}-ionophore, could be visualized as an attempt to reduce the calpain-1 activation and thus the digestion of its targets in these membrane structures, through the chaperone recruitment from cytosol. The **Fig. 11** shows the proposed model for the lipid rafts modification following the increase in $[Ca^{2+}]_i$.

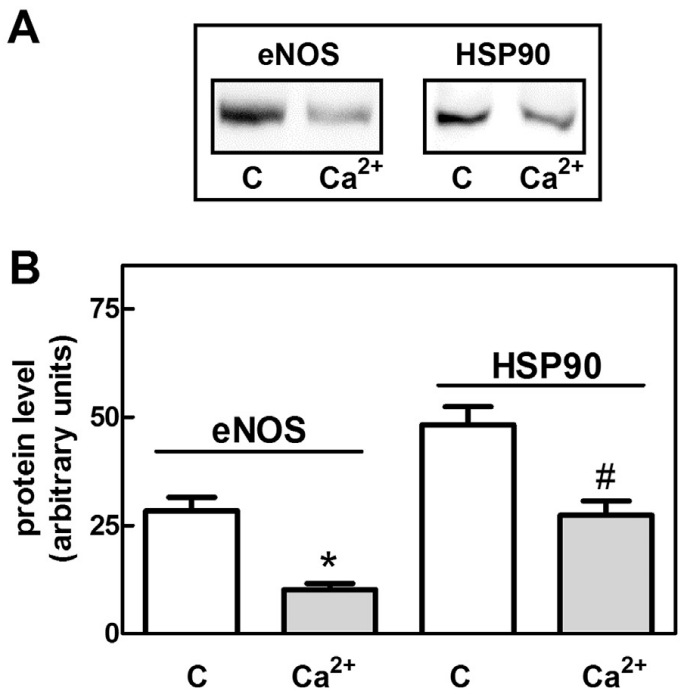

Figure 10: **Calpain-mediated digestion of eNOS and HSP90 in isolated lipid raft/caveolin-1 membrane microdomains.** (**A**) Gradient fraction 7 obtained as described in Methods was suspended in 120 µl of 50 mM sodium borate, pH 7.5 containing 1 mM EDTA and 0.5 mM 2-mercaptoethanol. Aliquots (50 µl) of the sample were incubated at 37 °C for 60 min in the presence (Ca^{2+}) or absence (C) of 1 mM Ca^{2+} and then were submitted to SDS-PAGE, followed by immunoblotting. eNOS and HSP90 were detected using the specific antibodies. The pictures represent one of the five experiments performed. (**B**) The immunoreactive bands detected were quantified. The reported values are means ± SEM of five independent experiments performed. * significantly different as compared to controls ($p = 0.0035$); # significantly different as compared to controls ($p = 0.0057$).

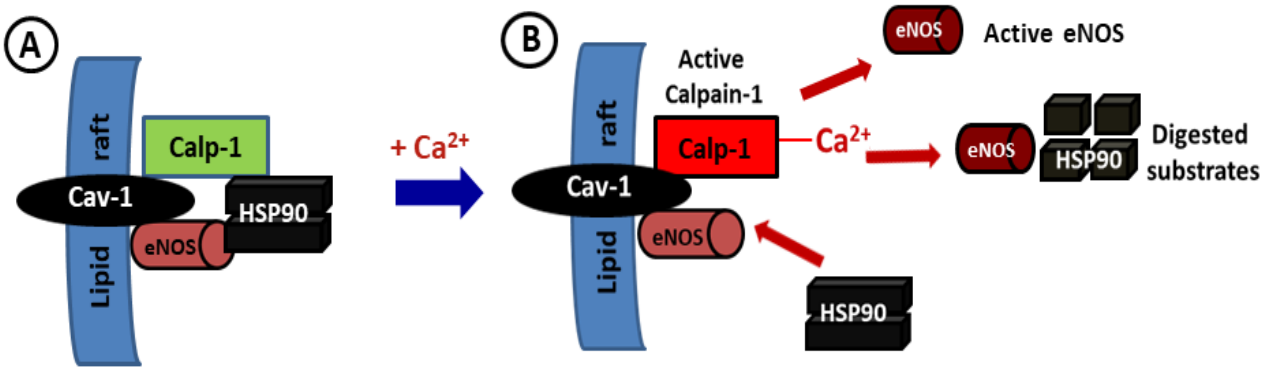

Figure 11: **Proposed model for the protein reorganization of the lipid raft.** (**A**) In resting cells, calpain-1 (Calp-1) is associated in an inactive form to membrane microdomains containing caveolin-1 (Cav-1), together with eNOS and HSP90. (**B**) In Ca^{2+} loaded cells, eNOS becomes active, dissociates from caveolae and translocates to cytosol, producing NO. Also calpain-1 becomes active and an intense or prolonged activation of the protease leads to the digestion of both eNOS and HSP90. However, in order to counteract the abnormal activation of calpain-1 and degradation of eNOS, HSP90 is recruited from cytosol.

4.4 Calpain-1 plays a protective role in endothelial cell

As reported so far, alteration of Ca^{2+} homeostasis leads to an alteration of the protein composition of caveolin-1 microdomain and of its function. It is conceivable that the protein rearrangement, occurring in lipid rafts, could represent a protective mechanism against Ca^{2+}-induced cell damages. However, although calpain-1 has been related to several pathological conditions, some reports have suggested that calpain-1 activity is involved in cell protection, especially against neurodegeneration [57, 58]. To investigate the role of calpain-1 in endothelial cell, the viability of bEnd5 cells was assayed, following appropriate treatments. bEnd5 cells, left untreated or treated with HG for 48 h, were exposed to 1 µM Ca^{2+}-ionophore for 2 h to cause 50 – 60% cell death. As shown in **Fig. 12**, cell death was reduced in cells previously treated with HG, suggesting that the calpain-1 activation, induced by exposure to HG, could have a positive effect. This hypothesis was supported by an increment in cell death when bEnd5 cells were treated with Ca^{2+}-ionophore, following exposure to HG in the presence

of CI-2 for 48 h, indicating that the calpain-1 inhibition increases cell damages, whereas its activation and the consequent controlled digestion of eNOS limit such damages. This fact was confirmed by a decrease in cell death when Ca^{2+}-ionophore exposure was carried out in the presence of a synthetic inhibitor of NOS, L-NMMA. However, cell death was also reduced if CI-2 was added only during the 2 h of Ca^{2+}-ionophore treatment. These results suggest that both the aberrant activation of calpain-1 and the over-activation of eNOS are responsible for endothelial cell damage. The first event can lead to a depletion of HSP90, whereas the second one promotes the over-production of NO and the possible consequent accumulation of ROS.

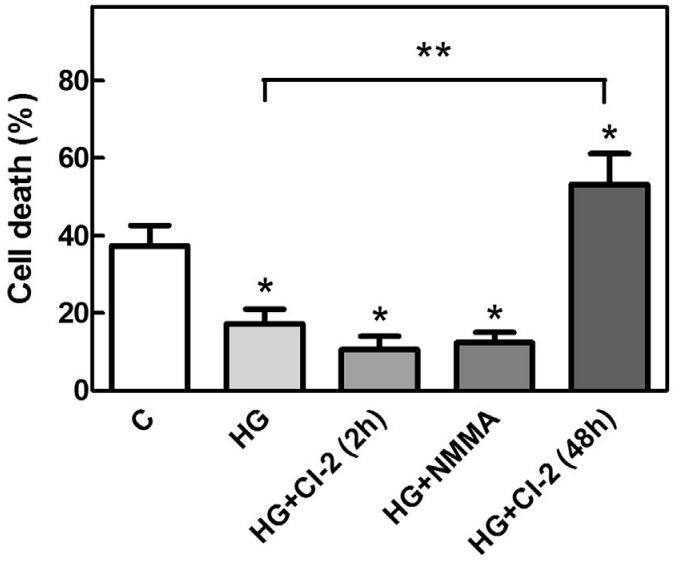

Figure 12: **Cell viability of bEnd5 cells in different conditions of intracellular Ca^{2+} overloading.** bEnd5 cells (10^4) incubated for 48 h in DMEM + 25 mM glucose in the presence of 2 µM CI-2 [HG+CI-2 (48 h)] were treated for 2 h with 1 µM Ca^{2+}-ionophore A23187. bEnd5 cells (10^4) incubated for 48 h in DMEM + 25 mM glucose were treated for 2 h with 1 µM Ca^{2+}-ionophore A23187 alone (HG), or in the presence of 2 µM CI-2 [HG+CI-2 (2 h)], or in the presence of 1 mM L-NMMA (HG+NMMA). As control (C), bEnd5 cells (10^4) were incubated for 48 h in DMEM and then treated for 2 h with 1 µM Ca^{2+}-ionophore A23187. After all incubations cell viability was evaluated by Neutral Red Uptake assay (see Methods). The values reported are the arithmetical mean ± SEM of six independent experiments performed. * significantly different as compared to controls ($p < 0.001$) according to ANOVA followed by post-hoc Dunnett's test; ** $p = 0.0005$ [HG vs HG+CI-2(48h)] according to t-test.

4.5 Alterations of the Ca^{2+}-proteolytic system in diabetic rat aorta

To assess whether the processes so far detected in an endothelial cell line occur also *in vivo*, the levels of calpain-1, eNOS and HSP90 were evaluated in aorta and brain cortex of ZDR, ZFR and ZLR animal models (see Materials and Methods). Rats tissues were processed and total lysates were submitted to immunoblotting, as reported in Methods. As shown in **Fig. 13**, in aorta of diabetic rats (ZDR), characterized by a significantly higher blood plasma glucose levels, the amounts of calpain-1, eNOS and HSP90 were considerably reduced as compared to lean rats (ZLR), used as controls, whereas in aorta of obese rats (ZFR), characterized by hyperinsulinemia and higher systolic arterial pressure, were poorly affected. These data suggest that in aorta of hyperglycaemic rats an intense calpain-1 activation occurs, resembling the digestion of lipid rafts proteins observed in endothelial cells following exposure to HG or Ca^{2+}-ionophore.

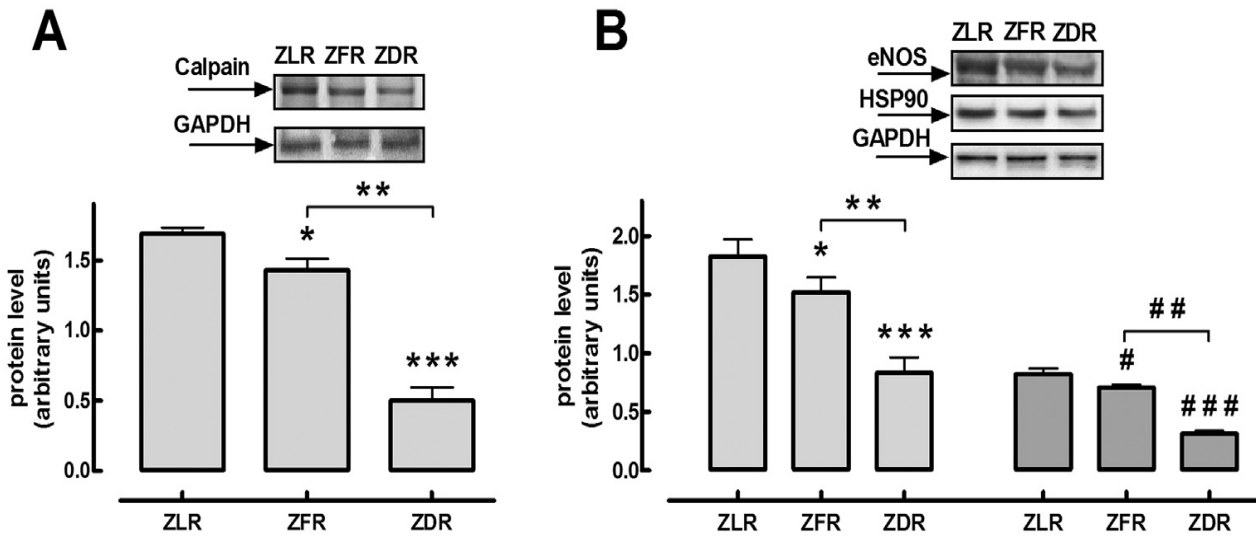

Figure 13: **Calpain-1, eNOS and HSP90 protein level in aorta of ZLR, ZFR, and ZDR.** (**A**) 50 µg or (**B**) 25 µg of proteins of crude extracts, prepared as described in Methods from aorta of ZLR, ZFR, and ZDR were submitted to SDS-PAGE and immunoblotting. (**A**) calpain-1, (**B**) eNOS (light grey bars) and HSP90 (dark grey bars) were detected using the specific mAbs. The immunoreactive material was developed and quantified. The images represent one of the experiments carried out. The reported values are means ± SEM of five independent experiments performed. Protein quantification was normalized versus the GAPDH immunoreactive band intensity, used as the loading control protein. (**A**) * significantly different as compared to ZLR ($p = 0.0469$); **$p = 0.0019$ (ZFR vs ZDR); *** significantly different as compared to ZLR ($p < 0.0001$). (**B**) * significantly different as compared to ZLR ($p = 0.0489$); **$p = 0.0206$ (ZFR vs ZDR); *** significantly different as compared to ZLR ($p = 0.0061$); # significantly different as compared to ZLR ($p = 0.0494$); ##$p < 0.0001$ (ZFR vs ZDR); ### significantly different as compared to ZLR ($p = 0.0003$).

In order to verify if these alterations induced by hyperglycaemia affect also other tissues, the amounts of calpain-1, HSP90 and the neuronal isoform of NOS, nNOS, were measured in brain cortex from ZDR, ZFR and ZLR. As reported in **Fig. 14**, the levels of calpain-1 and HSP90 in both diabetic and obese animals were comparable to that detected in lean control rats, whereas nNOS was slightly increased in ZDR. These results suggest that, although nNOS is a calpain-1 substrate, the fluctuations in Ca^{2+} homeostasis induced by hyperglycaemia and the consequent proteins alterations are tissue specific.

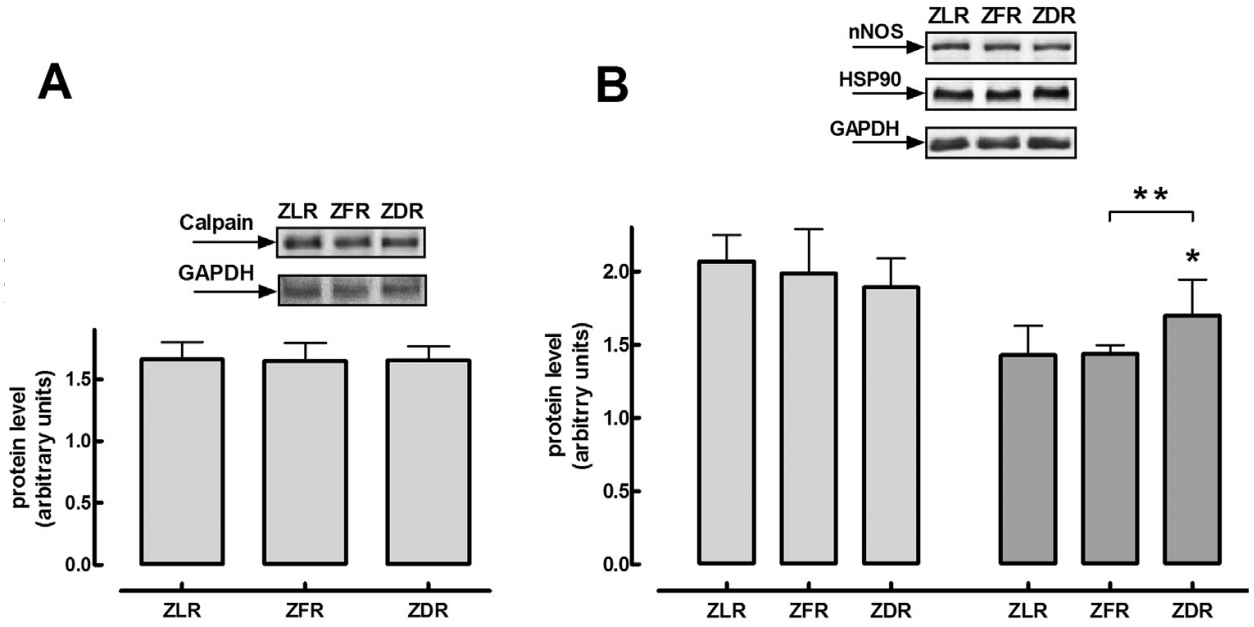

Figure 14: **Calpain-1, nNOS and HSP90 protein level in brain cortex of ZLR, ZFR, and ZDR.** (**A**) 50 µg or (**B**) 25 µg of proteins of crude extracts, prepared as described in Methods from brain cortex of ZLR, ZFR, and ZDR were submitted to SDS-PAGE and immunoblotting. (**A**) calpain-1, (**B**) nNOS (light grey bars) and HSP90 (dark grey bars) were detected using the specific mAbs. The immunoreactive material was developed and quantified. The images represent one of the experiments carried out. The reported values are means ± SEM of five independent experiments performed. Protein quantification was normalized versus the GAPDH immunoreactive band intensity, used as the loading control protein. (**B**) * significantly different as compared to ZLR ($p < 0.0001$); **$p < 0.0134$ (ZFR vs ZDR).

4.6 Calpain-1, inserted into multiprotein NMDAR complex, is associated to lipid rafts in SK-N-BE cells

As previously reported, calpain-1 is associated to the NMDAR multiprotein complex, together with HSP90 in resting SK-N-BE neuroblastoma cells [22]. As in bEnd5 cells, also in SK-N-BE cells calpain-2 and calpastatin are not detectable at this protein complex (see **Fig. 15**).

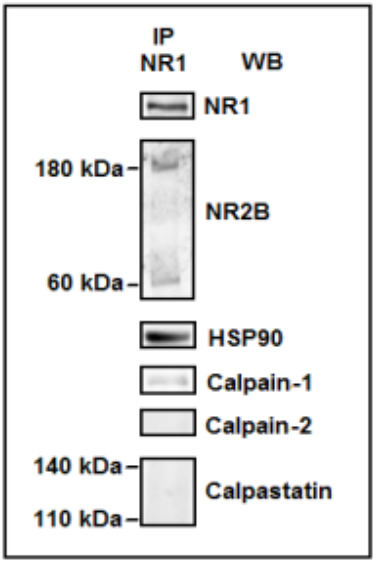

Figure 15: **Immunoprecipitation of the NR1 subunit in SK-N-BE cells.** The NR2B subunit, detectable as a 180 kDa native form and a 60 kDa digested form, HSP90 and calpain-1 are co-immunoprecipitated with the NR1 subunit. Adapted from [22].

Several studies have suggested that in nervous tissues NMDAR is associated to lipid rafts and this interaction can be modified during memory formation or by chemical agents [59 – 61]. However, there is very limited information about the possible association of calpain-1 and some of its substrates to membrane lipid rafts in cells of the nervous system. Thus, we evaluated if this association, already detected in endothelial cells, was present also in neuroblastoma cells, commonly used as an *in vitro* model in neurobiology [62]. Although caveolin-1, abundant in

endothelium, has been found in blood-brain barrier [63], it is not equally expressed in nervous tissues, where it is poorly detectable (see **Fig. 17 B** and **18 B**). Thus, EGFR, typically associated to membrane lipid rafts and particularly expressed in cells of nervous system, was chosen as marker of lipid rafts [35, 64]. Membrane lipid rafts were isolated from SK-N-BE cells following the same protocol used for bEnd5 cells and protein concentration was measured in each gradient fraction by the Lowry method (**Fig. 16 A**). After immunoblot analysis, the lipid rafts marker EGFR was recovered predominantly in the fraction 7, the same gradient fraction in which we previously detected caveolin-1 in bEnd-5 cells. In the same fraction we also recovered significant amounts of calpain-1, NMDAR subunits (NR1 and NR2B), HSP90 and the neuronal isoform of NOS (nNOS), whereas calpain-2 was not detectable in any fraction (**Fig. 16 B**).

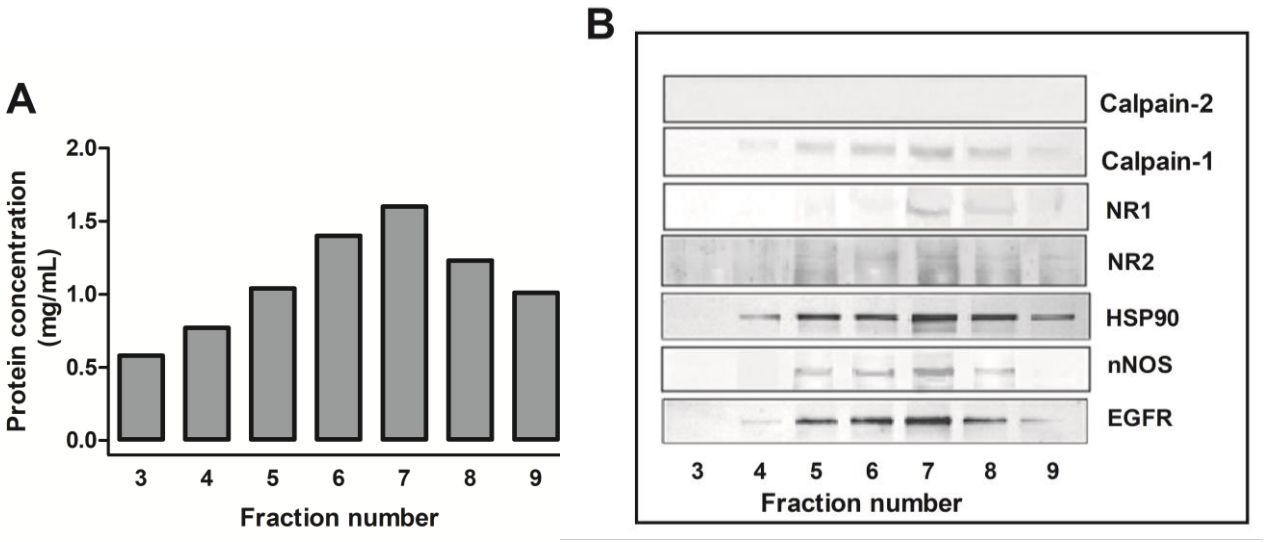

Figure 16: **Protein composition of lipid rafts isolated from SK-N-BE cells**. (**A**) SK-N-BE cells (35×10^6) were lysed to obtain lipid rafts as described in Methods. The protein concentration of each fraction was measured and only the values referred to fractions 3-9, containing measurable protein level, are reported. (**B**) Aliquots (30 µg) of each fraction were subjected to immunoblot analysis with the indicated antibodies. The pictures represent one of the three experiments performed.

4.7 Alteration in Ca^{2+} homeostasis modifies the protein composition of lipid rafts isolated from rat brain cortex

To obtain more information on the relationship between the lipid rafts structure and Ca^{2+} homeostasis and to study the possible lipid rafts reorganization caused by fluctuations in $[Ca^{2+}]$ in nervous tissue, we utilised, as an animal model, genetically hypertensive rats from the Milan strain, characterized by a constitutive alteration in $[Ca^{2+}]_i$ [65 – 67]. Particularly, we analysed the protein composition of lipid rafts isolated from brain cortex of normotensive and hypertensive rats from the same strain.

After the collection of the ten gradient fractions, protein concentration was measured and the values obtained from normotensive rats (see **Fig. 17 A**) showed a distribution similar to that measured in SK-N-BE cells. However, as shown in **Fig. 17 B**, the marker EGFR was found mainly among the fractions 5 and 7, also containing significant amounts of nNOS, whereas calpain-1, NMDAR subunits and HSP90 were recovered predominantly between fractions 6 and 7. As already mentioned, caveolin-1 was very poorly detectable, so it could not be used as a reliable lipid rafts marker in nervous tissue. Calpain-2 was not detectable, confirming the high degree of selectivity of the calpain-1 isoform in these membrane structures.

To verify if a chronic alteration in Ca^{2+} homeostasis could cause modifications and reorganization in lipid rafts, we isolated these membrane microdomains from brain cortex of hypertensive rats. Surprisingly, preliminary results highlighted that the distribution of lipid rafts and of the proteins of our interest among the fractions was profoundly different than that observed in normotensive animals (see **Fig. 18 B**). The marker EGFR was accumulated in lower density gradient fractions (fractions 3 and 4), where calpain-1 and HSP90 were absent. The levels and the localization of calpain-1 were not significantly affected in hypertensive animals, whereas HSP90 disappeared in fraction 7 and underwent a shift to fractions 8 and 9, although at lower amounts. NR1, NR2B and nNOS were distributed both in the lowest and

highest density fractions, but the total amounts of NR2B and nNOS, both calpain-1 substrates [22, 23, 27], were slightly decreased. It is interesting to note that also the protein concentration of the various fractions was significantly different from those obtained from normotensive animals (see **Fig. 17 A and 18 A**).

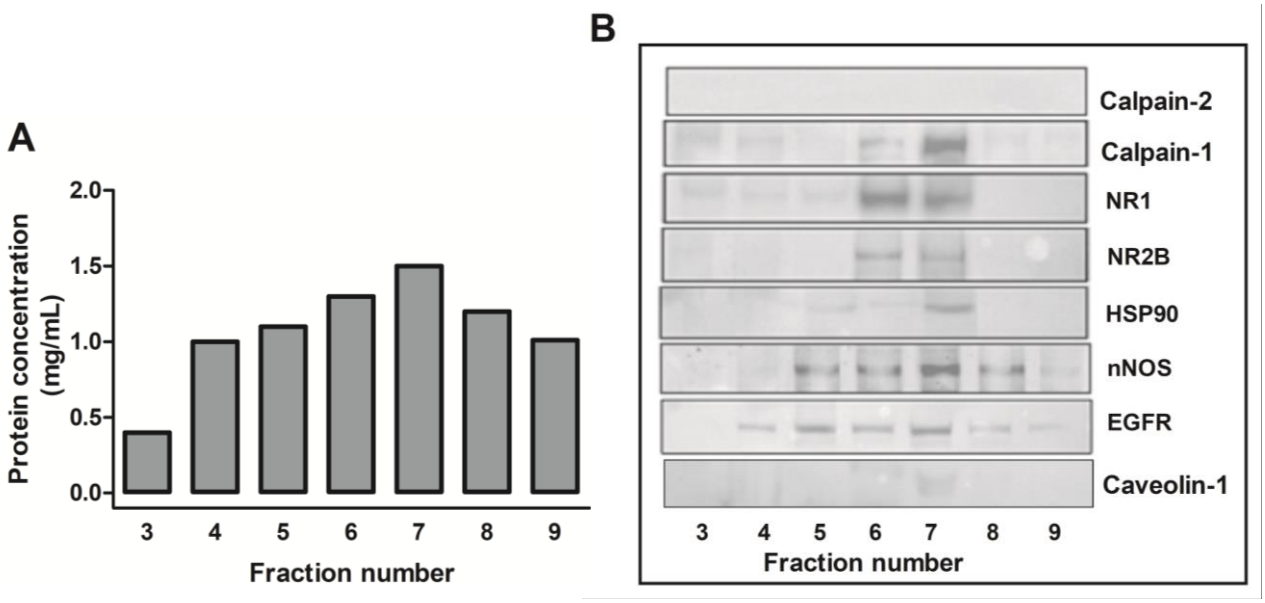

Figure 17: **Protein composition of lipid rafts isolated from normotensive rat brain cortex**. (**A**) Brain cortex samples were lysed to obtain lipid rafts as described in Methods. The protein concentration of each fraction obtained was measured and only the values referred to fractions 3-9, containing detectable protein amounts, are reported. (**B**) Aliquots (30 µg) of each fraction were subjected to immunoblot analysis with the indicated antibodies. The pictures represent one of the three experiments performed.

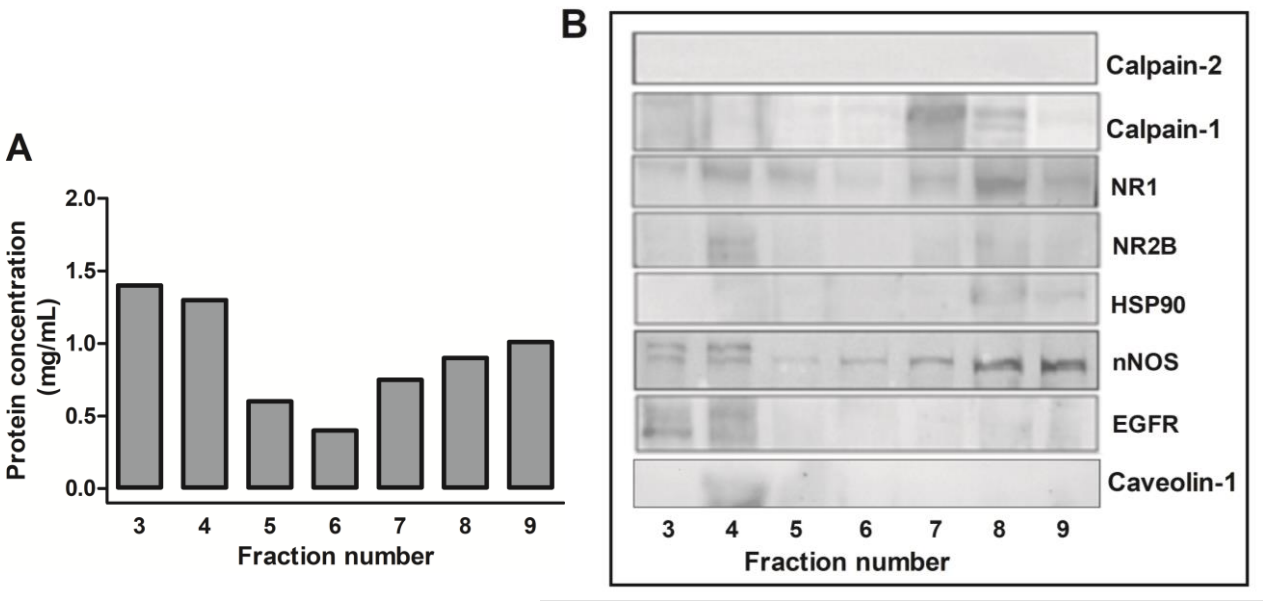

Figure 18: **Protein composition of lipid rafts isolated from hypertensive rat brain cortex**. (**A**) Brain cortex samples were lysed to obtain lipid rafts as described in Methods. The protein concentration of each fraction obtained was measured and only the values referred to fractions 3-9, containing detectable protein amounts, are reported. (**B**) Aliquots (30 µg) of each fraction were subjected to immunoblot analysis with the indicated antibodies. The pictures represent one of the three experiments performed.

To investigate if the protease resident in these membrane structures was responsible for digestion of its substrate also in nervous tissue, the gradient fraction containing the highest amount of both EGFR and calpain-1 (fraction 7), obtained from normotensive rat brain, was incubated with Ca^{2+} in order to activate the protease. As shown in **Fig. 19**, calpain-1 was slightly decreased (approximately 20%) by auto-proteolysis, whereas HSP90, one of calpain-1 substrates present in the same gradient fraction, was approximately 70% reduced. As expected, calpain-1 and HSP90 decrease was almost completely prevented if the treatment with Ca^{2+} was performed in the presence of the calpain-inhibitor-2, confirming that HSP90 reduction was due to a selective digestion mediated by calpain-1. Although further analyses are necessary to

hypothesize a possible role of calpain-1 in lipid rafts of rat brain cortex, altogether these preliminary results suggest that following activation of calpain-1 resident at the lipid raft, the digestion of proteins associated to these structures and their redistribution can be required for triggering the onset or sustain specific cell functions or can represent a mechanism for cell adaptation to the increase in $[Ca^{2+}]_i$.

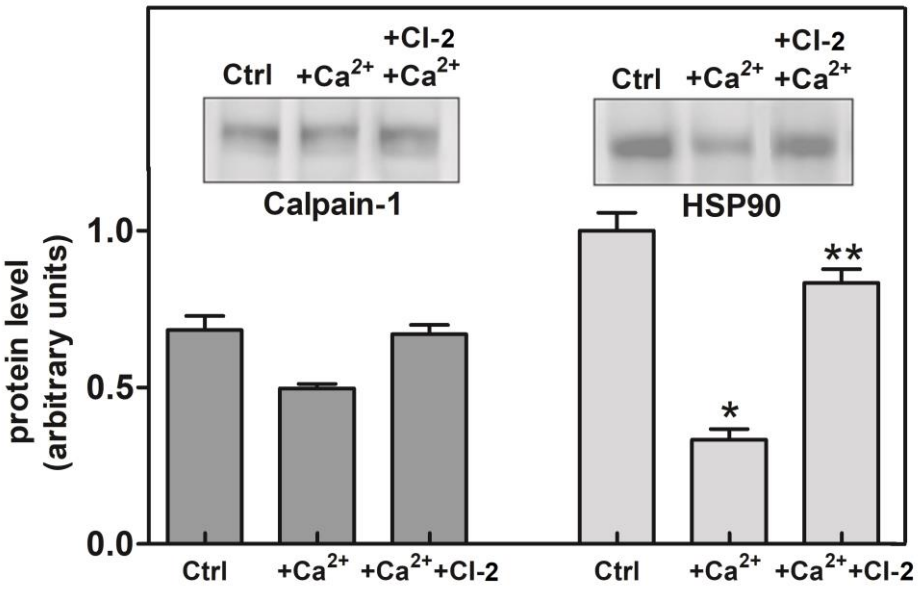

Figure 19: **Calpain-mediated digestion of HSP90 in isolated lipid rafts from normotensive rat brain.** Gradient fraction 7 obtained from brain cortex samples as described in Methods, was suspended in 150 µl of 50 mM sodium borate, pH 7.5 containing 1 mM EDTA and 0.5 mM 2-mercaptoethanol. Aliquots (50 µl) of the sample were incubated at 37 °C for 60 min in absence (Ctrl) or in presence (+Ca^{2+}) of 1 mM Ca^{2+} or, alternatively, in presence of 1 mM Ca^{2+} and 2 µM CI-2 (+Ca^{2+}+CI-2) and then were submitted to SDS-PAGE, followed by immunoblotting. Calpain-1 (dark grey bars) and HSP90 (light grey bars) were detected by using the specific primary antibodies and the immunoreactive bands were quantified. The pictures represent one of the three experiments performed. The reported values are means ± SEM of three independent experiments performed. * significantly different as compared to controls ($p < 0.05$); ** significantly different as compared to (+Ca^{2+}) ($p < 0.05$).

5. DISCUSSION

It has been reported that calpain-1, once intracellularly activated by a mobilization of calcium ions, translocates from the cytosol to the plasma membrane, where it may catalyse the limited digestion of its substrates, which predominantly consist of plasma membrane-associated proteins. [11, 13]. However this model seems insufficient to explain the selectivity of calpain-mediated digestion, since the protease does not have a specific consensus sequence [2, 68]. Here we have addressed our research starting from previous observations, obtained from us and from other Authors, that identified an aliquot of calpain-1 associated with the plasma membrane in resting cells [11, 26]. Thus, focusing on this calpain-1 localization, we have analysed if a selective interaction of the protease with a pool of specific proteins could contribute to regulate its catalytic activity and the molecular features responsible of its autocatalytic activation and sub-cellular localizations in resting and altered conditions of intracellular Ca^{2+} homeostasis.

It is important to note that we have isolated membrane lipid rafts following a new protocol, adapting different procedures carried out with both cells and ex vivo tissues. The insolubility of these membrane microdomains, rich in cholesterol and sphingolipids, in non-ionic detergents, such as Triton-X 100, has been previously widely exploited for their isolation. However, it has not been fully established that all rafts are detergent resistant. Moreover it has been shown that many detergent resistant membrane fractions unrelated to rafts can be obtained [69]. Particularly, the use of detergent can produce artifacts or interfere with glycolipids distribution in brain tissues [45]. Several lipid rafts-associated proteins, many of which have remained undetected for a long time [44], have been now identified by proteomics studies. However, interpretation of individual studies is limited by potential undefined contaminant proteins [70]. For these reasons, in the last two decades the protocols for the isolation of rafts have been directed toward the use of detergent-free methods, taking advantage of the unique buoyant density of membrane rafts [44]. Persaud-Sawin *et al* [45] reported an optimized detergent-free method for lipid rafts

isolation from brain tissue, compared to Triton-X 100-based method. It has been shown that the best yield is obtained in the absence of detergent. For our work, we developed a detergent-free protocol suitable for both cell lines and tissues, exploiting the typical density of lipid rafts. In future experiments, other lipid rafts markers might be used in addition to those that we have so far selected, to verify a possible overlap or divergence with other similar membrane structures.

Our results demonstrate that in endothelial and neuroblastoma cell lines as well as in rat brain cortex, lipid rafts membrane microdomains are the site of activation and action of calpain-1 associated with the plasma membrane. In the same localization we also detected NOS and HSP90. Moreover, in SK-N-BE cells and rat brain, these enzymes also interact with the NMDAR protein cluster. Although calpain-1 and calpain-2 share many protein targets of their proteolytic attack [11], the different roles played by the two proteases could be explained not only in the basis of the different Ca^{2+} requirement necessary for their activation *in vitro,* but also by a distinct cellular localization that promotes a peculiar accessibility to their relevant substrates. The results obtained in the present study are consistent with previous observations that attributed a different role to calpain-1 and calpain-2 towards single intracellular putative substrates. Specifically, the alternative silencing of both calpain-1 and calpain-2 isozymes in endothelial cells has demonstrated that degradation of eNOS depends only on calpain-1 activity, while calpain-2 regulates eNOS, promoting its phosphorylation [56]. Moreover, it has been shown very recently that calpain-1 deficient mice experienced reduced leukocyte adhesion to the aortic endothelium in response to myeloperoxidase, establishing a role for calpain-1, but not for calpain-2, in the endothelial dysfunction and vascular inflammation [71]. Conversely, VEGF/VEGFR2 induces calpain-2 dependent activation of PI3K/AMPK/Akt/eNOS pathway in endothelial cells and consequently the production of NO and a physiological angiogenesis [72]. In our experimental conditions, the absence of calpain-2 in the lipid rafts membrane microdomains makes the substrates accessible only to calpain-1.

The data obtained in bEnd5 cells indicate that a controlled but significant degradation of eNOS operated by calpain-1 is crucial to maintain proper amounts of active eNOS, preventing the accumulation of NO and the generation of Reactive Oxygen Species (ROS) which could damage cell structures [31]. The digestibility of eNOS and the extent of activation of calpain-1 are on the other hand modulated by HSP90 [24, 27, 28]. When the three proteins are located in the same cell structure and associated each other, eNOS digestion is strictly controlled. Indeed, the increase in the levels of HSP90 in the lipid rafts, following a stimulation with Ca^{2+}, can be interpreted as a mechanism for regulating the process of both autolytic calpain-1 activation and efficiency of its proteolytic activity towards the co-localized susceptible substrates. It is important to consider that, through this mechanism, calpain-1 can undergo regulation also in absence of its specific calpastatin inhibitor. As a result of the chaperone accumulation and protein reorganization in caveolin-1 microdomains, bEnd5 cells exposed to HG acquire a major resistance to Ca^{2+}-mediated insult, as revealed by cell viability assays. Cell viability probably depends on a balance between activation of calpain-1 and eNOS. An imbalance of these two events seems to shift the cell from a defensive or adaptive condition to an amplified and irreversible damaging conditions that leads to cell death. In this context, calpain-1 could play a protective role against NO overproduction, limiting the amount of eNOS protein.

Through the experiments performed in aortic endothelium of diabetic rats, it has been possible to prove that a prolonged perturbation of intracellular Ca^{2+} homeostasis can promote the transition from a cell defensive physiological condition to a pathological one. In aorta of diabetic rats, calpain-1, eNOS and HSP90 levels are significantly reduced compared to control animals and the alteration in the protein complex probably affects the interaction among the three proteins, causing a deficient control of the production of NO, necessary to preserve the endothelium functionality. Since in brain cortex of diabetic rats the three proteins remain completely unaffected, we can conclude that the alterations induced by hyperglycaemia are tissue specific. In fatty rat aorta, the levels of the three proteins are only slightly reduced,

indicating that in these animals the protein complex is poorly altered and calpain-1, eNOS and HSP90 are still operating in order to counteract the onset of endothelial dysfunctions.

The lipid rafts represent a specific site of activation of calpain-1 also in neuroblastoma cells and in nervous tissue, where the protease is selectively inserted in the NMDAR protein complex [22] and associated to lipid rafts/EGFR membrane microdomains also containing nNOS. Also this localization seems highly specific since calpain-2 is not detectable. In these regions of the plasma membrane, calpain-1 is involved in modulating the levels of both the glutamate receptor and nNOS, in order to control a toxic Ca^{2+} influx or an excessive NO production, due to a possible over-activation of these two proteins involved in Ca^{2+} mediated events [22, 23, 27]. In fact, it has been observed that digestion of the NR2B subunit and nNOS are strictly related to cell function and survival. Specifically, once digested, NR2B is rapidly internalized in inner membranes, preventing a damaging influx of Ca^{2+} [22].

In normotensive rat brain cortex, calpain-1 shows a distribution among the gradient fractions, obtained for the isolation of lipid rafts, similar to that observed in SK-N-BE cells. The partioning in lipid rafts probably improves the selectivity of calpain-mediated proteolysis towards its susceptible protein targets, preventing an aberrant and widespread action of the protease that could cause cell damages. The changes detected in the levels and in the gradient fractions distribution of the proteins analysed in brain cortex of genetically hypertensive rats suggest that when the protease is activated by a prolonged increase in $[Ca^{2+}]_i$, as it occurs in the tissues of these animals, the digestion of the chaperone HSP90 promotes a considerable lipid rafts reorganization, which probably allows a cell adaptation to the alteration of Ca^{2+} homeostasis, preventing an excessive calpain-1 activation. The results obtained from *in vitro* experiments on isolated lipid rafts, in which the resident calpain-1 is *in situ* activated by Ca^{2+} loading, demonstrate that both the autoproteolysis and the digestion of HSP90, are similar to those obtained in endothelial cells. The calpain-1 activation is probably, at least in part, restricted by a recruitment of HSP90 from cytosol. Altogether the experimental evidences included in this

study converge towards the notion of an important role played by the calpain-1-containing protein complex in lipid rafts. Thus, we consider that these are promising basis to unravel the role of calpain-1 in lipid raft dysfunction, accompanied by intracellular Ca^{2+} dysregulation. The full characterization of the mechanisms related to the recruitment of HSP90, in order to prevent a massive digestion of calpain-1 potential substrates, following alteration in $[Ca^{2+}]_i$, requires further experiments to correlate the calpain-1 activity to the changes in protein composition of the lipid rafts and to better define the regulation of the calpain-1 activation at this site.

It has been shown that neuronal intracellular Ca2+ signaling is abnormal in many neurodegenerative disorders including amyotrophic lateral sclerosis (ALS) [73]. Moreover lipid raft disturbances have been described in human motor neuron-like cells that express the mutated ALS protein SigR1 [74]. We have demonstrated previously that a transgenic mice model affected by amyotrophic lateral sclerosis shows elevation in $[Ca^{2+}]_i$ and activation of calpain-1 in the central nervous system compared to control animals [75]. Hence in the next future we will explore the lipid raft-associated protein pattern in different tissues from these transgenic mice during the progression of the disease, and the consequent possible modification of cell signalling. All the experiments will be carried out also on the tissues obtained from age matched control animals. In fact, the critical role of calpain-1 in the early phase and during the progression of ALS, suggests that the characterization of change in the lipid raft composition could identify new therapeutic targets able to counteract the onset and fatal course of this disease.

Note: Some of the data reported in this thesis have already been published: Ref. [76].

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