Endothelium microenvironment sensing leading to nitric oxide mediated vasodilation: nervous and biomechanical signals

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Blood vessels are continuously exposed to various stresses such as mechanical strains and neurosignals. Besides its role of barrier between blood and other tissues, the endothelium is a cell layer highly important for the regulation of vascular tone. Indeed, depending on the signal perceived by the endothelial cell, it can drive a vasoconstrictor or vasodilator signal. In this review, we will focus on the mechano-receptors and neuro-receptors which lead to the vessels relaxation via the production of nitric oxide. In a second part, we will give some evidence of a potential cross-talk between these two kinds of perception.

Introduction

Different types of vessels such as arteries and veins exist in the vascular system. All vessels are composed by three distinct layers whose organization is characteristic of a particular type of vessel. The most inner layer called the tunica intima is composed by the vascular endothelium, which acts as a barrier with blood. The tunica media is composed by smooth muscle cells and elastic fibers. This layer performs the vasomotor activity (vasoconstriction or vasodilation) and is regulated by the sympathetic innervation. The most outer layer called the tunica externa is composed principally by collagen fibers to increase interaction with underlying tissues and strengthen vessels. In veins these three strata are in contact whereas in arteries they are separated by the internal and the external elastic lamina. Endothelium is anchored on a basal lamina and the sub-endothelial layer, which is an extracellular matrix (ECM) allowing a mechanical slipping on others structures. The vasoactivity of vessels is critical for the regulation of blood pressure [1].

A vessel is not a “simple pipe”, but a highly dynamic structure imbricated in a proper environment. It allows a precise physiological adaptation to the variations of pH, temperature, mechanical forces or neurosignals. The endothelium can communicate with smooth muscle cells to elaborate a vasoactive answer via endothelium derived relaxing factors (EDRF) such as nitric oxide (NO) and prostaglandin I2 (PGI2) [2].

The production of NO by the endothelium is mediated by endothelium nitric oxide synthase (eNOS) which catalyses NO and L-citrullin production from L-arginin. eNOS is initially sequestrated at the plasma membrane by caveolin-1 and sequentially activated by interactions with partners (Figure 1). The action of calcium (Ca2+) and calmodulin (CaM) activate this enzyme in a calcium-dependent phase. Then, the association with HSP90 protects eNOS against proteosomal degradation, and Akt from phospho-inositide 3-kinase (PI3K) pathway can phosphorylate eNOS on serines in a calcium-independent phase to maintain NO production. PKA is also able to phosphorylate eNOS. If the stimulus is prolonged, eNOS mRNA can be transcribed and stabilized [3].

There are different ways to activate this eNOS in endothelial cells. We will first review the mechanical sensing inducing the eNOS activation, then the neuro-sensing inducing the eNOS activation, and finally we will try to evidence a potential cross-talk between these pathways with the impact of the mechanical environment on neuro-sensing.

Mechanical sensing

It is well known that mechanical stimuli can enhance eNOS activation via different cellular pathways (see Table 1). Blood pressure, circumferential stretch (tension) and shear stress are the three manifestations...
of mechanical stress in vessels [4]. Shear stress, the most studied stress, is referenced to activate various mechanical receptors. It is perceived at the apical membrane and can be transmitted into the cell and at the basal membrane via the cytoskeleton.

**Caveolae**

They are invaginated islets of the membrane where lots of receptors and channels are concentrated. The main molecule in these structures is caveolin-1 which is enshrined in the cell membrane and allows deformation. Although its structural role and its capacity to sequester eNOS at the plasma membrane [3], caveolin-1 has been shown as a necessary component to activate the mitogen associated protein kinase (MAPK) / extracellular signal-regulated kinase (ERK) signaling pathway [5]. Moreover, mechanotransduction via β1 integrins also requires these structures [6]. As a consequence, caveolae can be considered as mechano-sensing micro domains, which convert the stress into a signalisation pathway.

**Receptor tyrosine kinases (RTK).**

Vascular endothelium growth factor Receptor 2 (VEGFR2) also known as Fetal Liver Kinase 1 (Flk-1/KDR) has been referenced to act as a mechanosensor of shear stress. Shear stress can activate the association between Flk1 and Shc, an adaptor protein to mediate a MAPK pathway via Ras [7]. Moreover the MAPK pathway promotes eNOS transcription [8]**. Shear stress can activate the dimerization of the receptor VEGFR2 by a mechanism which is not understood and bind Src to activate the PI3K pathway too [9].

**G Protein Coupled Receptor (GPCR)**

Chachisvilis et al. showed that the Bradykinin 2 (B2) G Protein Coupled Receptor (GPCR) can be activated by shear stress in a ligand-independent manner via a conformational transition induced by a membrane dynamic process [10]. The link has not been clearly demonstrated yet, but it is well established that B2 receptor activates a phospholipase C (PLC) to increase the intracellular calcium and activate eNOS [11,12]. So we can hypothesize that B2 GPCR activation leads to NO production.

**Cytoskeleton and adhesions**

Many studies have reported mechanical stress as a way to alter the tensegrity model, which represents the equilibrium conformation of the cytoskeleton. The reorganization of cytoskeleton after mechanical stress induces clustering and activation of many molecules including adhesion complexes such as integrins and cadherins [13]*. The rupture of this tensegrity equilibrium by shear stress can activate two types of junctions: adherens junctions and focal adhesions which link actin network with others cells, and basal membranes.

Endothelial adherens junctions are composed by a high variety of proteins including vascular endothelial cadherin, VEGFR2 and Platelet Endothelial Cell Adhesion Molecule 1 (PECAM1). Tzima et al showed that shear stress is able to activate PECAM1 by

### Table 1. Types of mechano-sensors implied in the mechanical stress response

<table>
<thead>
<tr>
<th>Mechano-sensor</th>
<th>Type of molecule</th>
<th>Inducer</th>
<th>Pathway engaged</th>
<th>Cell</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caveolae</td>
<td>Caveolin</td>
<td>SS</td>
<td>MAPK</td>
<td>BAEC</td>
<td>[5]</td>
</tr>
<tr>
<td>TRPV4</td>
<td>Ion channel</td>
<td>SS, cytoskeleton (?)</td>
<td>Ca**++/ Ca**++ exit</td>
<td>RCAECs arteries</td>
<td>[26]</td>
</tr>
<tr>
<td>KCa</td>
<td>Ion channel</td>
<td>Intracellular Ca**++</td>
<td>K** exit</td>
<td>HUVEC</td>
<td>[27]</td>
</tr>
<tr>
<td>KIR</td>
<td>Ion channel</td>
<td>Membrane hyper-polarization</td>
<td>K** exit</td>
<td>BAEC</td>
<td>[27]</td>
</tr>
<tr>
<td>Cal**+ channel</td>
<td>Ion channel</td>
<td>Membrane hyper-polarization</td>
<td>Cal**+/ Cal**+/ Ca**++ exit</td>
<td>HUVEC</td>
<td>[27]</td>
</tr>
<tr>
<td>Outward rectifying Cl channel</td>
<td>Ion channel</td>
<td>SS, cytoskeleton (?)</td>
<td>Membrane depolarization</td>
<td>HAEC</td>
<td>[27]</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>RTK</td>
<td>SS</td>
<td>MAPK</td>
<td>BAEC</td>
<td>[7]</td>
</tr>
<tr>
<td>Bradykinin B2 Receptor</td>
<td>GPCR</td>
<td>SS (ligand-independent)</td>
<td>PLC ([Cal**+] increase)</td>
<td>BAEC</td>
<td>[9]</td>
</tr>
<tr>
<td>Adherens junctions (VE-Cad, VEGFR2, PECAM1)</td>
<td>Adhesion molecules, RTK</td>
<td>SS, cytoskeleton (?)</td>
<td>MAPK</td>
<td>PI3K</td>
<td>BAEC</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesions</td>
<td>SS</td>
<td>MAPK</td>
<td>BAEC</td>
<td>[16]**</td>
</tr>
<tr>
<td>Src</td>
<td>Focal adhesions</td>
<td>SS</td>
<td>eNOS stabilization</td>
<td>BAEC</td>
<td>[8]**</td>
</tr>
<tr>
<td>PAK</td>
<td>Kinase</td>
<td>Rac/Cdc42 on fibronectin</td>
<td>NfkB</td>
<td>BAEC</td>
<td>[19]</td>
</tr>
<tr>
<td>αβ1-integrin (only?)</td>
<td>Focal adhesions</td>
<td>SS, cytoskeleton (?)</td>
<td>PI3K</td>
<td>Mesenteric resistance arteries</td>
<td>[22]</td>
</tr>
<tr>
<td>Glycocalyx</td>
<td>Glycoproteins</td>
<td>SS</td>
<td>Alteration of the tensegrity equilibrium</td>
<td>Femoral artery of farm pig, BAEC</td>
<td>[23,24]</td>
</tr>
<tr>
<td>Isolated G Protein</td>
<td>G proteins</td>
<td>SS</td>
<td>liposomes</td>
<td></td>
<td>[25]</td>
</tr>
</tbody>
</table>

phosphorylation, which plays the role of a mechanical transducer to activate Src kinase [14]**. The action of Src to phosphorylate VEGFR2 is facilitated by vascular endothelial cadherin, which acts as an adaptor. In this context, this RTK can phosphorylate PI3K to activate eNOS.

On the other side, focal adhesions are mediated principally by integrins that link the extracellular matrix with the actin network via a protein complex including vinculin, talin, tensin, paxillin and alpha-actin [15]. Moreover, integrins bind many proteins involved in signaling like focal adhesion kinase (FAK), Src kinase, Fyn kinase and p130. It has been shown that shear stress is able to regulate eNOS mRNA transcription and stabilization. Indeed, shear stress activates rapidly PI3K phosphorylation involved in the activation of MAPK / ERK and c-Jun N-terminal kinase (JNK) pathways to promote eNOS transcription [8,16]**. Furthermore, Src is activated by shear stress too and stabilize eNOS mRNA for a longer answer [8]**.

But shear stress has other effects mediated by integrins. Tzima et al. noticed that the activation of αVβ3 integrin in response to blood flow leads to a cytoskeletal alignment in the direction of flow [17]. Shear stress can also activate integrin singling via other intermediate complexes. It is the case of adherens junctions where shear stress activates PI3K and further induces integrins activation depending on the nature of bound ECM protein. It was demonstrated by Orr et al. that on a collageneic matrix, PI3K activates α2β1 integrins, and on a fibronectin substrate it activates αVβ3 and αVβ1 integrins [18]**. Moreover, the activation of a specific integrin inactivates the other kind of dimer. This suppression is mediated by the activation of Protein kinase A (PKA) by α2β1 integrin which suppresses αVβ3 integrin activation, and Protein Kinase C (PKC) by αVβ3 integrin which suppresses α2β1 integrin activation. This clearly demonstrates that the composition of the ECM influences the cellular answer.

In addition, shear stress is able to activate p21-activated kinase (PAK) in cells grown on a fibronectin substrate via Rac/Cdc42 but not on collagen. PAK activates the Nuclear Factor-kappaB (NF-kappaB) transcription factor known to activate pro-inflammatory genes, and to increase permeability and eNOS transcription [19,20]. Moreover, (PKA) stimulated by integrins in contact with collagen inhibits the activation of PAK and so of NF-kappaB [19,21].

It was also shown that the flow-induced vasodilatation is highly decreased when α1β1 integrins are blocked by an anti-α1β1 antibody. These integrins, which bind collagen and laminin are sensitive to shear stress and activate eNOS via a PI3K/Akt pathway [22].

**Membrane system**

Different components associated with the plasma membrane such as glyocalyx and isolated G-proteins can play a role of mechano-transducer. Glyocalyx is a cell coating including lots of molecules: glycoproteins such as sialic acids (SA), proteoglycans and glycosaminoglycan such as heparan sulfate (HS), chondroitin sulfate (CS) and hyaluronic acid (HA). Specific degradation of HS, HA or SA results in a loss of the shear stress-induced NO production. As the glyocalyx is connected with the cytoskeleton, authors proposed that shear stress could be able to alter the tensegrity equilibrium via glyocalyx mechano-activation [23,24]**. Isolated G-protein has been also reported as a potent role for shear stress transduction. Indeed, it is known that isolated G-proteins are found at the plasma membrane. Normally inactive because of the absence of receptor, Gudi et al. showed that shear stress can activate these proteins [25]. Shear stress can change membrane properties by decreasing micro-viscosity and increasing fluidity. This leads to a different lipid dynamic able to isolated G-protein independently of a receptor. G-proteins are involved in lots of pathway to increase NO production, so we can assume that the activation of these isolated proteins can lead to the same result.

**Ion channels**

The activation of Ion channels in a context of shear stress acts at different moments. They can be activated directly by shear stress such as Transient Receptor Potential cation channel subfamily V member 4 (TRPV4), or after an intracellular calcium increase. TRPV4 channels can be activated by shear stress to allow the entrance of extracellular calcium [26]. This influx participates at the activation of eNOS on a calcium-dependent phase and activates calcium activated potassium channel (KCa), which allows K⁺ exit. This outward leads to a hyper-polarization of the cellular membrane, which increases both conductance of inward rectifying potassium channel (Kir) and the opening of calcium channels sensitive to the hyper-polarization. The opening of Kir channels increases the hyper-polarization which auto-amplifies the phenomenon. Moreover, shear stress increases the opening probability of Kir channels. In parallel, outward rectifying chloride channel are open just after potassium channels, probably to maintain the polarization of the plasma membrane. Calcium can be delivered by the endoplasmic reticulum too, for example after the action of PKA which is implied in lots of pathways [3,7,28].

Barakat et al. showed that shear stress can be transmitted by different ways from vessel lumen to endothelial cells. Flow can directly activate channels at the plasma membrane; but it can deform this membrane too, which transmits this stress to channels. Cytoskeleton, after shear stress forces can modulate the activity of this channels too [29].

A particularly interesting finding is the fact that TRPV4, which was described to answer to shear stress, is also able to answer to acetylcholine (Ach) [30]**. This activation by a neuropeptide is mediated by a pathway including a GPCR to sense Ach and a PKC alpha to transduce the information [31]**. This observation led us to think that it could exist an interaction between mechanical and neuro-sensing by the endothelial cell to induce NO production.
Neuro-sensing

A vessel is a structure integrated in the organism. As a consequence, it communicates with others organs via lots of molecules such as neuropeptides, cytokines and chemokines. Molecules arrive via the blood flow or by sympathetic nerves from the tunica media. Here, we review the neuropeptides which lead to eNOS activation and the NO production (see Table 2).

Acetylcholin

Whereas acetylcholin (Ach) acts as a vasoconstrictor in the absence of endothelium, it promotes a vasodilation via an EDRF when it is perceived by the endothelial cell. Ach, which is commonly considered as a neuropeptide, acts via a muscarinic receptor on the endothelium. The binding of this receptor leads to a conformational change which activates a Gq protein coupled with a muscarinic receptor. Gq-α subunit is known to activate a PLC which produces inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2). IP3 can bind the IP3-Receptor on the endoplasmic reticulum membrane to release Ca$^{2+}$ and further activate CaM and eNOS to produce nitric oxide [32].

Calcitonin Gene Related Peptide (CGRP)

CGRP was identified in 1983 as a neuropeptide [33]. Two years after, it was described as a potent vasodilator implied in inflammatory reactions [34]. CGRP can be released by the sympathetic innervation and picked up at the membrane of endothelial cells to transmit a nervous message. CGRP is able to bind CGRP-Receptor which is composed by two molecules: a GPCR with seven transmembrane domains called Calcitonin receptor-Like Receptor (CRLR) associated with the Receptor Activity-Modifying Protein 1 (RAMP1). A CRLR not associated with RAMP cannot work as a functional CGRP receptor [35,36].

It appears that CGRP could act via two distinct ways following the activation of a GPCR. The binding of the receptor favors the exchange of a GDP for a GTP on α subunit to activate targets. The first way, which is not clearly demonstrated, is a PLC-dependent pathway activating a CaM/Ca$^{2+}$-dependent NO production as described for Ach. Evidences shows that the activation of the CGRP receptor can activate adenylate cyclase at the plasma membrane by the βγ subunits. This enzyme is able to transform ATP into cAMP which increases in concentration and leads to the activation of PKA also known as cAMP dependent protein kinase (cAK). PKA can activates AMPK which is probably the intermediate that allows eNOS phosphorylation and its activation in a CaM/Ca$^{2+}$ independent way [36–38]**.

Kinin

Kmins are neuropeptides produced from kininogen by kallikrein enzyme and are particularly important for cardiovascular regulation. There are two main kinins: bradykinin and kallidin. They are two plasma neuropeptides that can interact with endothelium via Bradykinin Receptor 2 (BR2) to modulate the vascular tone. Even if the principal kinin is Bradykinin, Kallidin can act on the endothelium too via the same receptor, BR2, coupled with different G-proteins, and as a consequence can activate lots of pathways [12]. The principal is the Gq Protein Coupled BR2 which activates PLC and releases intracellular calcium to increase eNOS activity as described for acetylcholin. It can activate GqPCR or GasPCR known to inhibit and activate adenylate cyclase respectively. G-Protein Coupled BR2 can activate MAPK/ERK pathway. Gq activates PLC whereas βγ activates PI3K. The Akt/PI3K pathway phosphorylates directly eNOS on Ser1177 which is a mark known to activates NO production [11,12].

Substance P

Substance P (SP) is a neuropeptide from trachykinin family which acts at different levels. It is particularly important for the automatic nervous system and the regulation of cardio-respiratory system. It is known to be a strong vasodilator. SP is delivered by the sympathetic innervation and acts on endothelial cells via the Neurokinin-1 Receptor (NK1-R). This NK1-R is a GqPCR which activates PLC and increases the level of eNOS activity via a Ca$^{2+}$/CaM-dependent manner [39]. Neurokinin A, another peptide of trachykinin family, can bind NK1-R too, but with a lower affinity.

Table 2. Types of neuropeptides involved in the neuro-vasodilatory response

<table>
<thead>
<tr>
<th>Neuropeptide</th>
<th>Receptor</th>
<th>Type of Receptor</th>
<th>Pathway engaged</th>
<th>Cell</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGRP</td>
<td>CGRP-R (CRLR+RAMP1)</td>
<td>GPCR</td>
<td>AC - PKA - AMPK</td>
<td>Rabbit, HUVEC</td>
<td>[36,38]**</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>Muscarinic receptor</td>
<td>GPCR</td>
<td>(?) PLC – IP3 - [Ca$^{2+}$] increase</td>
<td>-</td>
<td>[36]</td>
</tr>
<tr>
<td>Bradykinin &amp; Kallidin</td>
<td>Bradykinin Receptor 2</td>
<td>GPCR</td>
<td>PLC – IP3 - [Ca$^{2+}$] increase, TRPV4 opening via PKC</td>
<td>-</td>
<td>[31,32]</td>
</tr>
<tr>
<td>Substance P &amp; NKA with lower affinity</td>
<td>NK1-R</td>
<td>GPCR</td>
<td>PLC – IP3 - [Ca$^{2+}$] increase</td>
<td>-</td>
<td>[11,12]</td>
</tr>
<tr>
<td>Agelin</td>
<td>APJ Receptor</td>
<td>GPCR</td>
<td>PLC – IP3 - [Ca$^{2+}$] increase, Akt, HUVEC</td>
<td>-</td>
<td>[39]</td>
</tr>
</tbody>
</table>

Apelin

It is a neuropeptide identified in 1998 by Tatemoto et al. [40]. It can be found in blood circulation and is implied in lots of physiological regulations such as blood pressure decrease. Apelin acts on the endothelial cell surface via APJ receptor which is a GPCR. The activation of GqPCR activates PLC via its α subunit to increase eNOS activity via calcium as described previously, whereas GiPCR activates Akt pathway by phosphorylation. This active form of Akt phosphorylates eNOS to highly increase its activity [41].

Other non neuro-peptides have been identified to acts as potent vasodilator via NO such as angiotensin II which induce BK-Receptor to avert an excessive vaconstriction, histamine during allergies, 5-HT (serotonin) and thrombin liberated during platelet aggregation, insulin, etc...

Neuro-mechano-sensing interplay

We saw in the two previous parts mechano- and neuro-sensing by the endothelial cell which lead to the production of NO and relax the underlying smooth muscle cells (Figure 2). It showed us that endothelium is a highly active structure which communicates with its environment.

It is interesting to notice the variability and the similarities of signal transduction mechanisms between the two types of reception. For neuro-sensing, all pathways tend to converge on intracellular calcium increase to activate eNOS in a CaM/Ca²⁺ dependent manner. Only apelin was referenced to act in part on Akt by promoting its phosphorylation, and CGRP which activates eNOS via PKA in a CaM/Ca²⁺ independent way. Moreover, only GPCRs were referenced to sense a neuropeptide and transduce an intracellular signal principally via PLC as an intermediate of transduction. Complementary, for mechano-sensing there is much more mechanisms implying eNOS activation,
transcription and its mRNA stabilization. They tend to PI3K phosphorylation and intracellular Ca\(^{2+}\) increase. As described in figure 1, because of the different ways engaged, we can expect a different response in term of amplitude and rapidity of NO production. Indeed, even if different pathways co-exist, a cell exposed to mechanical stress activates rapidly PI3K/Akt pathway to promote NO synthesis and smooth muscle cell relaxation. If the stimulus is maintain for a longer period, there is eNOS mRNA transcription via MAPK and Nfkb. For a neuropeptide response, there is only one pathway engaged, which is proportional at the intracellular calcium concentration. The pathway is activated as long as the receptor is engaged with its ligand. This allows a rapid switch between activation and inhibition of eNOS. Even for neuropeptides, the quick reversibility of the response is modulated as observed for BK and KD which are able to activate both calcium concentration increase and MAPK to stimulate eNOS mRNA transcription. Another thing we can remark is the importance of cytoskeleton for mechano-sensing. Shear stress induces a break of the tensegrity equilibrium, which allows clustering of signaling complexes (such as adherens junctions, focal adhesions and RTK dimerization) and the opening of ion channels. On the opposite, neuro-sensing seems independent of cytoskeleton in term of stress transduction.

Although great differences exist regarding the pathway engaged and the type of reception, these observations show the presence of some common intermediaries between mechano- and neuro-sensing consistent with a potential cross-talk. Indeed, some evidences in the scientific literature show that the two phenomenons described previously can act together.

As we described previously, TRPV4 channels can be activated both by shear stress and acetylcholin [30,31]. It shows us that two types of stress which are perceived by two types of receptor can converge via the same protein. We can hypothesize that the activation of one way modulates the activation of the other.

Another illustration of this potential cross-talk between mechano- and neuro-sensing is the pressure-induced vasodilation (PIV) [42]**. An external pressure on skin activates a neuron circuit set into dermis. The consequence is the transduction of a nociceptive information via capsaicin-sensitive fibers to spinal cord. As a consequence, a nervous message is elaborated on skin activates a neuron circuit set into dermis. The consequence is the transduction of a nociceptive information via capsaicin-sensitive fibers to spinal cord. As a consequence, a nervous message is elaborated via the ability of PIV ?

We must keep in mind that only vasodilator signals were presented here, however there are lots of vasoconstrictor signals too. As a consequence, vessels are in a permanent equilibrium to maintain a good physiological blood pressure and local irrigation.

Acknowledgments

I thank Dr Romain Debret and Dr Laurent Viriot for their help and their advices concerning this review.

References and recommended reading

Papers of particular interest have been highlighted as:

- of special interest
- of outstanding interest


This paper shows that shear stress can activate c-Src to promotes on the one hand eNOS transcription via ERK pathway and on the other hand eNOS mRNA stabilization.

Ecole Normale Supérieure de Lyon BioSciences Master Reviews, Dec. 2013
This paper demonstrates the importance of the sub-
endothelial extracellular matrix composition to promotes
a specific integrin response. Moreover, the activation of a
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This paper is the first which identifies an in vivo link between a vasodilation mediated by acetylcholine and TRPV4 channels involved in mechanosensing.


This paper is one of the first which identify the pathway Ach-PKCα-TRPV4.


This paper consolidate the hypothesis of PKC implication under CGRP perception. Moreover, they integrate the AMPK as a novel intermediate in the CGRP-PKA-eNOS pathway.


By using in vivo methods, this article was the first to describe the pathway of pressure induced-vasodilation. It includes the CGRP as a central neuropeptide to promote the physiologic vasodilatory reflex.