Chapter

# L-Arginine/Nitric Oxide Pathway and KCa Channels in Endothelial Cells: A Mini-Review

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## Abstract

The endothelium is an organ with a key role in the maintenance of cardiovascular health through the regulation of vascular tone, vascular resistance, blood flow, and arterial pressure. These functions are related with the synthesis and release of vasoactive molecules, mainly vasodilators like nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF). Both factors are released and diffused from endothelial cells to the smooth muscle cells, where there is a subsequent activation of signaling pathways that finally decrease the intracellular calcium to induce the vascular relaxation. The study of the molecular mechanisms that underlie the endothelial function still is in development, but from the evidence obtained from the endothelial cells *in vitro* studies are possible to partially describe the pathways to regulate the physiological endothelial function and the disturbances in pathological conditions. In this mini-review, we describe the main mechanisms for NO synthesis and the role of potassium channels related with EDHF. We include schemes and graphical summaries for better understanding of the molecular regulation of vascular tone in the human cardiovascular system.

**Keywords:** L-arginine, nitric oxide, potassium channels, endothelium

## 1. Characteristics of the endothelium

The endothelial cells (ECs) have mesenchymal origin, length of 25–50 μm and form a flat epithelium called endothelium. The endothelium in a human adult is composed of approximately 1–6 × $10^{13}$ cells, constituting an organ that weighs approximately 1 kg and covers a surface area of approximately 1–7 $m^2$ [1]. For decades, the endothelium was considered as a simple barrier between blood and the rest of the body's tissues. However, since the early 1980s, this vision changed radically [2] and, today, the endothelium is considered a true organ that fulfills multiple functions in the physiology and pathophysiology of vascular system, including autocrine, paracrine, and endocrine actions and the regulation of coagulation and fibrinolysis processes [3].

One of the most important functions of endothelial cells is their participation in the regulation of vascular tone. In the classic article of Furchgott and Zawadzki in 1980, it was demonstrated that the presence of the endothelium is essential for the vasodilator effect induced by acetylcholine in isolated blood vessels pre-constricted with norepinephrine. In those years, it was proposed that the vasodilation was

produced through a factor that was released by the endothelium in response to agonists [4]. This factor was called the endothelial-derived relaxing factor (EDRF) [5]. Between 1986 and 1990, it was concluded that this factor corresponded to nitric oxide (NO) [6, 7]. The endothelium responds to mechanical stimuli such as pressure and flow stress ("shear stress"), hormonal stimuli, and vasoactive substances that regulate the vascular tone. The endothelial cells release molecules that regulate vasomotor function, inflammation, and hemostasis. Vasodilators agents include NO, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF). Vasoconstrictors agents include endothelin 1, angiotensin II, thromboxane A2, and reactive oxygen-derived species (ROS). Inflammatory mediators include NO, intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1), E-selectin, and NFκB (**Figure 1** [8]).

Since the discovery of NO, the mechanisms of endothelial cell activation and endothelial dysfunction have been studied. In this way, the quiescent endothelial cells express a vasodilator, anticoagulant, and anti-adhesive phenotype, while the activated endothelial cell expresses procoagulant, pro-adhesive, and vasoconstrictive properties [9]. It has been considered that the decrease in the capacity of the vascular endothelium to stimulate vasodilation generates endothelial dysfunction, a phenomenon that is observed in several pathological conditions such as hypertension, hypercholesterolemia, diabetes mellitus, hyperhomocysteinemia, chronic kidney failure, chronic heart failure, etc. Although the molecular basis for endothelial dysfunction is not fully understood, numerous studies point to decreased biosynthesis and/or NO activity as a central mechanism [10–13].

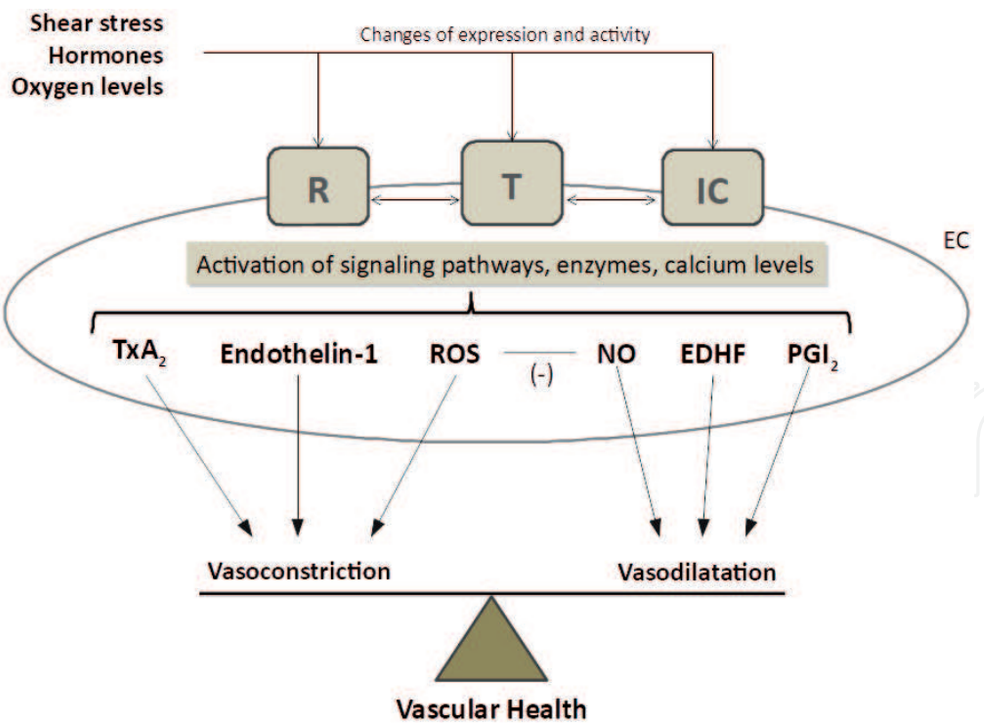


**Figure 1.**
*Vascular tone regulation. The vascular tone is partially regulated by the local factors secreted by endothelial cells (ECs) in response to physical factors like shear stress and humoral and chemical factors like hormones and oxygen levels. The changes in blood flow are detected by membrane proteins, mainly receptors (Rs), transporters (Ts), and ion channels (ICs). There is a network connecting the activities of these proteins through signaling pathways that induce the release of different mediators like thromboxane A2 (TxA2), endothelin 1, reactive oxygen species (ROS), nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), or prostacyclin (PGI2), among others. The equilibrium between the vasoconstrictors and vasodilators factors maintains the endothelial function and vascular health.*

## 2. Synthesis of nitric oxide in the endothelium

NO is synthesized from the semi-essential cationic amino acid L-arginine, which must be transported from the extracellular space into the endothelial cell by a family of cationic amino acid transporters (CATs) [14]. This amino acid is the substrate in a reaction where the metabolic product corresponds to L-citrulline in an equimolar proportion with the coproduct NO [15, 16]. This reaction is catalyzed by the enzyme NO synthase (NOS), which can be classified into their constitutive forms (cNOS) and their inducible form (iNOS) [17]. The cNOS includes the endothelial isoform (eNOS) and the neuronal isoform (nNOS), both producing NO in short bursts at low concentrations (nM) and in a calcium-dependent manner to fulfill the physiological functions of NO. The physiological activity of eNOS is dependent on several cofactors and is regulated by signaling pathways that induce phosphorylation in different sites for activation (serine 1177) or inhibition (threonine 495) [17]. NO diffuses from endothelial cells to smooth muscle cells (SMCs) and activates the soluble guanylate cyclase (sGC) pathway, to reduce the intracellular calcium and induce vasodilation (**Figure 2**). iNOS is mainly expressed in cells that participate in the inflammatory response after induction by cytokines and other inflammatory mediators, producing NO in high concentrations (μM) and independently of calcium [18–20].

The availability of NO in vivo is regulated by a combination of NO synthesis and inactivation. The decrease in the availability of NO may be due to a lower expression

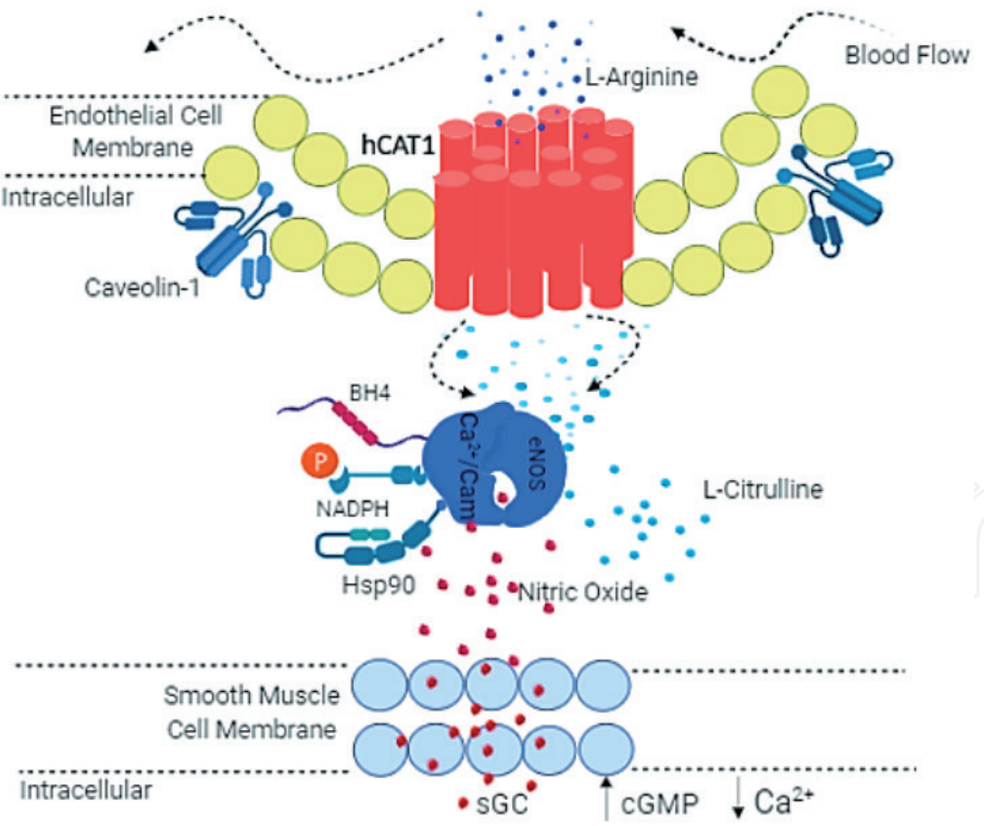


**Figure 2.**
*L-arginine transport and nitric oxide synthesis in endothelial cells. hCAT-1 is a protein expressed in plasma membrane of endothelial cells, mainly in plasma membrane invagination called caveolae. The L-arginine enters to the cell from blood and is used by eNOS to synthesize L-citrulline and nitric oxide (NO). The eNOS needs different cofactors to maintain its function, which include tetrahydrobiopterin ($BH_4$), nicotidamine adenine dinuclotide phosphate (NADPH), and heat shock protein 90 (Hsp90). Nitric oxide diffuses through the cell membranes and enters the smooth muscle cells to activate the soluble guanylate cyclase (sGC). The sGC synthesizes cyclic GMP (cGMP), which activates protein kinase G and, after subsequent steps, the intracellular calcium decreases to induce the vasodilation.*

or activity of eNOS, as a result of the action of endogenous and exogenous inhibitors or due to the lower availability of the substrate L-arginine [8, 14]. The availability of NO can also be diminished by the rapid reaction between NO and reactive oxygen-derived species (ROS) [13].

## 3. Reactive oxygen-derived species (ROS) in endothelium

Endothelial cells generate ROS, including the superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide ($H_2O_2$), peroxynitrite ($ONOO^-$), hydroxyl radical ($^{\cdot}OH$), among others [15, 16]. In endothelial cells, the main sources of ROS are the enzymatic complex xanthine oxidoreductase (XOR) [17], the complex of membrane nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) [18], eNOS itself when it is "uncoupled" due to lack of tetrahydrobiopterin ($BH_4$) or L-arginine [19], mitochondrial cytochromes [20], and hemoglobin [21].

Among all endothelial ROS sources, NADPH oxidases are enzymes whose primary function is the generation of ROS and they play an important role in redox signaling [22]. On the other hand, the activity of NADPH oxidase can cause the uncoupling of eNOS by the oxidative degradation of $BH_4$, leading to the eNOS-dependent synthesis of $O_2^{\cdot -}$ and detriment of the synthesis of NO [18, 23]. Once $O_2^{\cdot -}$ is synthesized, it can act as a precursor to other ROS due to its use by superoxide dismutase (SOD) to generate $H_2O_2$ that has greater stability and capacity to cross biological membranes, and it therefore can act as a modulator of signal transduction pathways [24]. Furthermore, $O_2^{\cdot -}$ reacts quickly with NO to generate $ONOO^-$, a powerful oxidizing agent that causes DNA fragmentation and lipid oxidation [25].

It is currently postulated that the mechanism by which $O_2^{\cdot -}$ "kidnaps" NO would play a central role in the development of endothelial dysfunction that is seen in pathologies such as diabetes mellitus [26–28], preeclampsia [29, 30], and hypertension [31].

## 4. L-arginine transport in human fetal endothelium

The amino acid L-arginine is taken up by endothelial cells through the transporter systems $y^+$, $y^+L$, $b^{0,+}$, and $y\,B^{0,+}$ [32–35]. Of these systems, there are two that have been described in HUVEC, that is, $y^+$ system [36–38] and $y^+L$ system [39]. The $y^+$ system family is currently known to include at least five cationic amino acid transporters (CATs) called CAT-1, CAT-2A, CAT-2B, CAT-3, and CAT-4. CAT-1 is expressed ubiquitously, CAT-2A and CAT-3 are constitutively expressed in liver and brain, respectively, while CAT-2B is induced in a variety of cell types in response to bacterial endotoxins and pro-inflammatory cytokines [40, 41]. CAT-4 corresponds to a cDNA sequence with 41–42% identity with the other members of the CATs family, but its transport activity has not yet been determined [32, 34, 35]. CAT-1, CAT-2B, and CAT-3 are characterized by high affinity to the substrate ($K_m$ = 100–400 μM) and independency of $Na^+$, while CAT-2A has low affinity for cationic amino acids ($K_m$ = 2–5 mM). Two members of CATs have been reported to be expressed in HUVEC, that is, hCAT-1 and hCAT-2B, while hCAT-2A and hCAT-3 transporters have not been detected in this cell type [34, 36–39] (**Table 1**). Although the hCAT-1 and hCAT-2B transporters have similar kinetic characteristics, it is possible to differentiate them by their different sensitivities to L-lysine trans-stimulation. In *Xenopus laevis* oocytes injected with hCAT-1 and hCAT-2B mRNA, L-lysine increases L-arginine transport by 9.8-fold and 1.8-fold, respectively [42]. Thus, for L-lysine trans-stimulation assays in HUVEC, it has been possible to determine that

| Gene | Protein | $K_m$ (μM) | Distribution |
|---|---|---|---|
| *SLC7A1* | CAT-1 | 70–250 | All tissues except liver and lacrimal gland |
| *SLC7A2* | CAT-2A | 2.2–5.2 | Liver, skeletal muscle, and pancreas |
| *SLC7A2* | CAT-2B | 38–380 | Endothelium, and inducible in several tissues |
| *SLC7A3* | CAT-3 | 40–120 | Thymus, ovary, testes, and brain |
| *SLC7A4* | CAT-4 | — | Brain, testes, and placenta |

*Proteins CATs are coded in different genes (except CAT2A and 2B, same gene), have different kinetic constants for the transport of L-arginine ($K_m$) and distribution in tissues.*

**Table 1.**
*CATs' family members.*

the hCAT-1 transporter accounts for 60–80% of the total uptake of L-arginine in physiological conditions [36–38]. The importance of the hCAT-1 transporter in NO synthesis has been confirmed through a transgenic mouse model that overexpresses the protein exclusively in the endothelium. Aortic rings obtained from these transgenic mice have a higher sensitivity to relaxation in response to acetylcholine compared to native mice, while endothelial cell cultures obtained from these animals, that overexpress hCAT-1, exhibit a greater NO synthesis [43].

## 5. Regulation of the expression of hCAT-1

Regarding the gene organization of CAT transporters, it is known that the *SLC7* family is phylogenetically composed of two subfamilies formed by cationic amino acid transporters (CATs) and glycoprotein-associated amino acid transporters (HATs). The cationic amino acid transporter family is encoded by the *SLC7A* (1–4) genes and corresponds to proteins with 14 transmembrane domains [44]. Specifically, the gene that encodes the hCAT-1 protein corresponds to *SLC7A1* whose open reading frame is formed by 11 exons and 10 introns. The gene is located on chromosome 13q12-13q14 [45].

Among the genes encoding CAT-1 in rat, mouse and human have common characteristics: the promoter region lacks TATA box, and they have multiple binding sites for the transcription factor specific protein 1 (Sp1) and they have an extensive 3′ non-translatable region (3′UTR) that could perform functions in the regulation of mRNA stability or in translation [46–49]. In rats, stress by amino acids deprivation induces an increase in the rCAT-1 mRNA expression by a mechanism related to increased mRNA stability [46]. This increased mRNA stability would be related to the presence of a regulatory region within the 3′UTR sequence of the gene [47]. Subsequent experiments have shown that the effect of amino acids deprivation on rCAT-1 expression would depend on both transcriptional [48] and posttranscriptional mechanisms [50].

In humans, it is known that insulin increases leg blood flow in healthy subjects via stimulation of endothelial NO synthase (eNOS) [51]. Insulin also increases the synthesis and release of NO and release in primary cultures of HUVEC [38, 52]. Biological effects of insulin involve activation of several transcription factors, including Sp1 in several cell types [53, 54]. Insulin increases Sp1 nuclear protein abundance and its binding to a proximal region (−177 and −105 bp from ATG) of the *SLC7A1* promoter containing four consensus sequences for Sp1 [55]. Interestingly, in patients with essential hypertension, a reduction of *SLC7A1* transcriptional activity due to reduced Sp1 activity in the promoter region has been reported [12]. So, the transcriptional regulation of *SLC7A1* is relevant for cardiovascular physiology,

and the reduction of the promoter activity of this gene could be associated with cardiovascular disease (CVD).

On the other hand, the first intron of *SLC7A1* may play a bifunctional role in regulating the *SLC7A1* transcriptional activity by the binding of the purine-rich element binding protein A (Pur alpha) in physiological conditions and by activating the transcription factor 4 (ATF4) in endoplasmic reticulum stress or by decreasing the *SLC7A1* transcriptional activity by the C/EBP homologous protein 10 (CHOP) binding in C6 rat glioma cells [56].

For the physiological regulation of hCAT-1 activity, both transcriptional regulation of *SLC7A1* and/or posttranscriptional regulation of *SLC7A1* transcript are relevant for the protein expression and L-arginine transport [55]. Insulin increases the expression of *SLC7A1* gene due to an increased transcriptional activity, most likely due to higher Sp1 activity. So, hCAT-1 expression and activity are regulated by insulin in endothelium, suggesting that in insulin resistance there is a reduction of L-arginine transport and NO synthesis that contributes to endothelial dysfunction and cardiovascular diseases.

## 6. High D-glucose and expression and activity of L-arginine/NO pathway

Hyperglycemia and diabetes mellitus are pathological conditions associated with fetal endothelial dysfunction [55] and type 2 diabetes mellitus (T2DM) [57] or cardiovascular disease (CVD) [58]. CVD in patients with diabetes mellitus is associated with the generation of ROS.

High concentration of D-glucose (25 mM) increases L-arginine transport and cGMP accumulation in endothelium in a similar manner to that observed in HUVEC from pregnancies with gestational diabetes [33, 59]. Increased L-arginine transport in response to incubation with high D-glucose has been related to increased mRNA levels for the hCAT-1 and eNOS activity in HUVEC [60]. In human aortic endothelial cells, prolonged incubation (7 days) with 25 mM D-glucose induces a decrease in eNOS activity (determined by nitrite content), protein abundance, and mRNA level. This effect is associated with a decrease in eNOS promoter activity [61]. In bovine aortic endothelial cells (BAECs), there is a lower production of insulin-induced NO when the cells were incubated with high extracellular concentration of D-glucose, an effect that seems to depend on a signaling pathway that involves to the type 1 insulin receptor (IR-1), phosphatidyl inositol 3 kinase, and the inhibitor of nuclear factor kappa-B kinase [62]. On the other hand, the increase of cGMP production induced by high D-glucose in HUVEC is blocked by incubating the cells with 1 nM insulin [63]. Incubation with 1 nM insulin (8 h) has been shown in this same cell type to be sufficient to block the effect that D-glucose has on the decreased transport of adenosine [64], an important vasoactive nucleoside [65].

In HUVEC, high extracellular D-glucose increases L-arginine transport, NO synthesis, and $O_2^{\cdot -}$ generation through eNOS and NADPH oxidase activation. Additionally, high D-glucose increased the contractile response in the human umbilical vein. Insulin reversed these effects of high D-glucose, leading to normal hCAT-1 expression, NO synthesis, ROS generation, and vascular tone. Insulin acts like antioxidant molecules (like tempol, ascorbic acid) to restore high D-glucose-increased oxidative stress in the fetoplacental vascular bed [66]. High D-glucose increases L-arginine transport, likely resulting from higher hCAT-1 expression and protein abundance in the plasma membrane. This mechanism could be an adaptive response of HUVEC to higher ROS generation from high D-glucose-activated

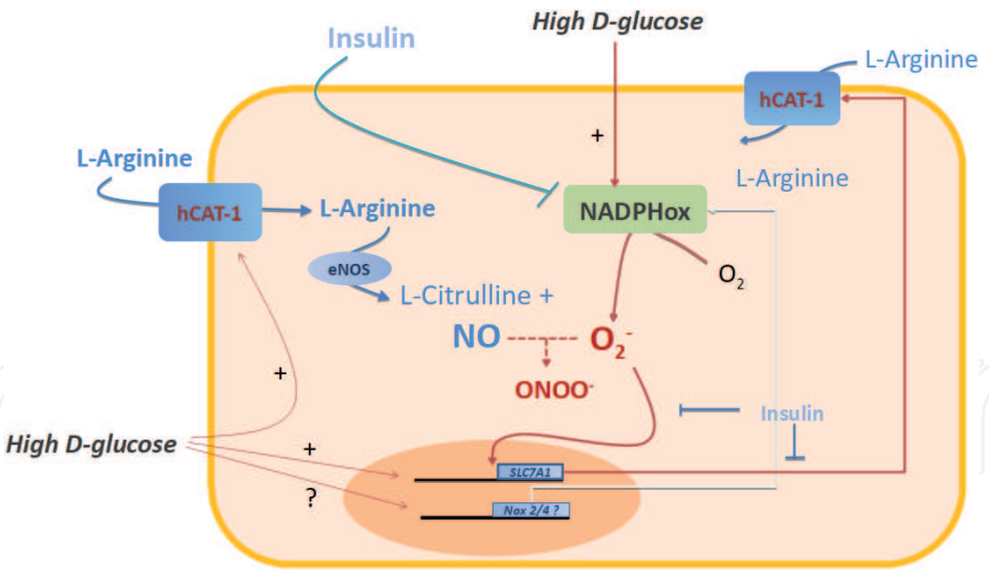


**Figure 3.**
*Endothelial dysfunction induced by high D-glucose and protection by insulin in HUVEC. Exposure of HUVEC to high D-glucose leads to an increase (↑) in the plasma membrane abundance of the human cationic amino acid transporter 1 (hCAT-1) and higher L-arginine uptake. High D-glucose activates NADPH oxidase, leading to higher generation of ROS, including $O_2^{\cdot-}$. Insulin restores ROS and $O_2^{\cdot-}$ generation to values in cells exposed to 5 mM D-glucose (normal), resulting in the restoration of hCAT-1-mediated L-arginine transport and nitric oxide (NO) synthesis. High D-glucose and insulin also activate the* SLC7A1 *promoter region (coding for hCAT-1) up to −650 bp from the ATG via a mechanism involving ROS and $O_2^{\cdot-}$ generation. In addition, insulin restores hCAT-1 protein abundance and its distribution in the cells via an NADPH oxidase-independent mechanism (data from González et al. [66]).*

NADPH oxidase. In parallel, high D-glucose increased NO synthesis. Insulin reversed the high D-glucose-mediated alterations in L-arginine transport involving the modulation of *SLC7A1* gene expression, leading to altered umbilical vein reactivity. Modulation of hCAT-1 expression and activity by insulin is the key to maintaining umbilical vein tone and endothelial function in physiologic and pathophysiological conditions (**Figure 3**) [66].

## 7. Role of potassium channels in endothelial function

Another important mechanism that regulates the endothelial function is the activity of ion channels that modulate the cell membrane potential. The calcium-activated potassium channels (KCa) have been shown to be relevant to induce the necessary hyperpolarization to stimulate the relaxation of vascular smooth muscle cells (related with EDHF). In systemic circulation, large conductance KCa (BKCa) channels have been shown preferentially expressed in VSMC, meanwhile small (SKCa) and intermediate (IKCa) conductance KCa are preferentially expressed in endothelium [67, 68]. However, potassium currents inhibited by iberiotoxin (BKCa inhibitor) have been described in HUVEC stimulated by sildenafil or insulin [69]. In fact, insulin (10 nM) can directly activate native and recombinant BKCa currents in cell-attached patch-clamping experiments with a rapid effect that is MAPK-dependent when the hormone was added in the pipette [70]. There is evidence that insulin may induce endothelial cell hyperpolarization by modulating K channels activity [38, 71]. The insulin-induced relaxation in human placental veins (~368 μm diameter), pre-constricted with U46619, is a mechanism dependent on the BKCa channel activity. The co-incubation of vessels with genistein (tyrosine kinases inhibitor) and wortmannin (PI3K inhibitor) did not block the insulin's relaxation, and by contrast potentiated the insulin-induced

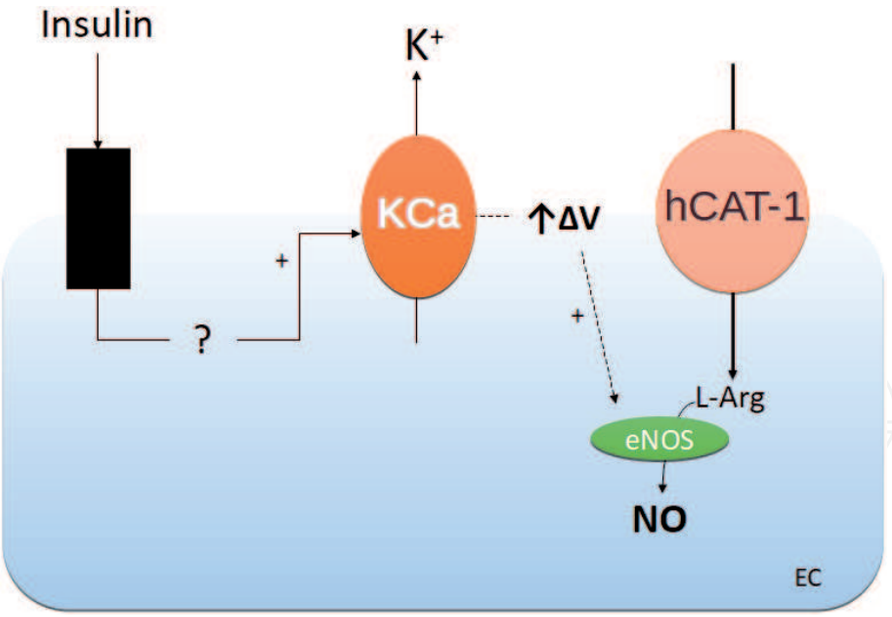


**Figure 4.**
*Proposal of mechanism for KCa activation by insulin. Evidence obtained in endothelial cells (ECs) shows that insulin activates KCa (mainly BKCa) in a mechanism still not fully understood. The activation of KCa by insulin induces hyperpolarization (↑ΔV), leading to activation of eNOS for NO synthesis from L-arginine uptake by hCAT-1 (modified from Rojas et al. [71]).*

vasodilation. Also, insulin decreased perfusion pressure (34 ± 3%) in the isolated cotyledon of normal placenta with a basal perfusion pressure of 64 ± 5 mmHg (or pre-constricted with U46619) [72]. The effects of insulin on BKCa activity are associated with evidences that show that the constriction induced by U46619 and $H_2O_2$ in placental vasculature is partially decreased with 10 nM insulin preincubation (10 min) in a mechanism totally dependent of BKCa activity [72]. Recently, it has been determined that insulin-mediated NO synthesis requires the participation of both IKCa/ BKCa channels and eNOS activity in HUVECs [71]. In the same cell type, insulin increased the open probability (NPo) of BKCa, associated with hyperpolarization in single cell analysis [69]. In human placental arteries, the relaxation induced by the NO donor, SNAP, is partially blocked by charybdotoxin (BKCa inhibitor) and almost totally blocked by charybdotoxin and ODQ (sGC inhibitor) [73]. Therefore, an extracellular stimulus that increases the NO availability activates a mechanism that involves sGC and BKCa activities [71]. These findings constitute evidence for postulating a new mechanism induced by insulin in human vasculature related with the physiological regulation of KCa activity for NO synthesis (**Figure 4**).

## 8. Final remarks

The relevance of the endothelium for cardiovascular physiology is well established, mainly by findings related to the capacity of endothelial cells to synthesize NO and regulate the plasma membrane potential of smooth muscle cells. **Figure 5** shows a graphical summary of the L-arginine/NO pathway in the human blood vessels that highlight the capacity of endothelial cells to respond to extracellular stimuli and translate the mechanical forces and endocrine signals to intracellular mechanisms leading to NO synthesis and activation of potassium channels. It is important to note that the subcellular distribution of hCAT-1 and eNOS is also relevant for endothelial cells function. In physiological state, hCAT-1 colocalizes with caveolin-1 in the plasma membrane caveolae in proximity to eNOS.

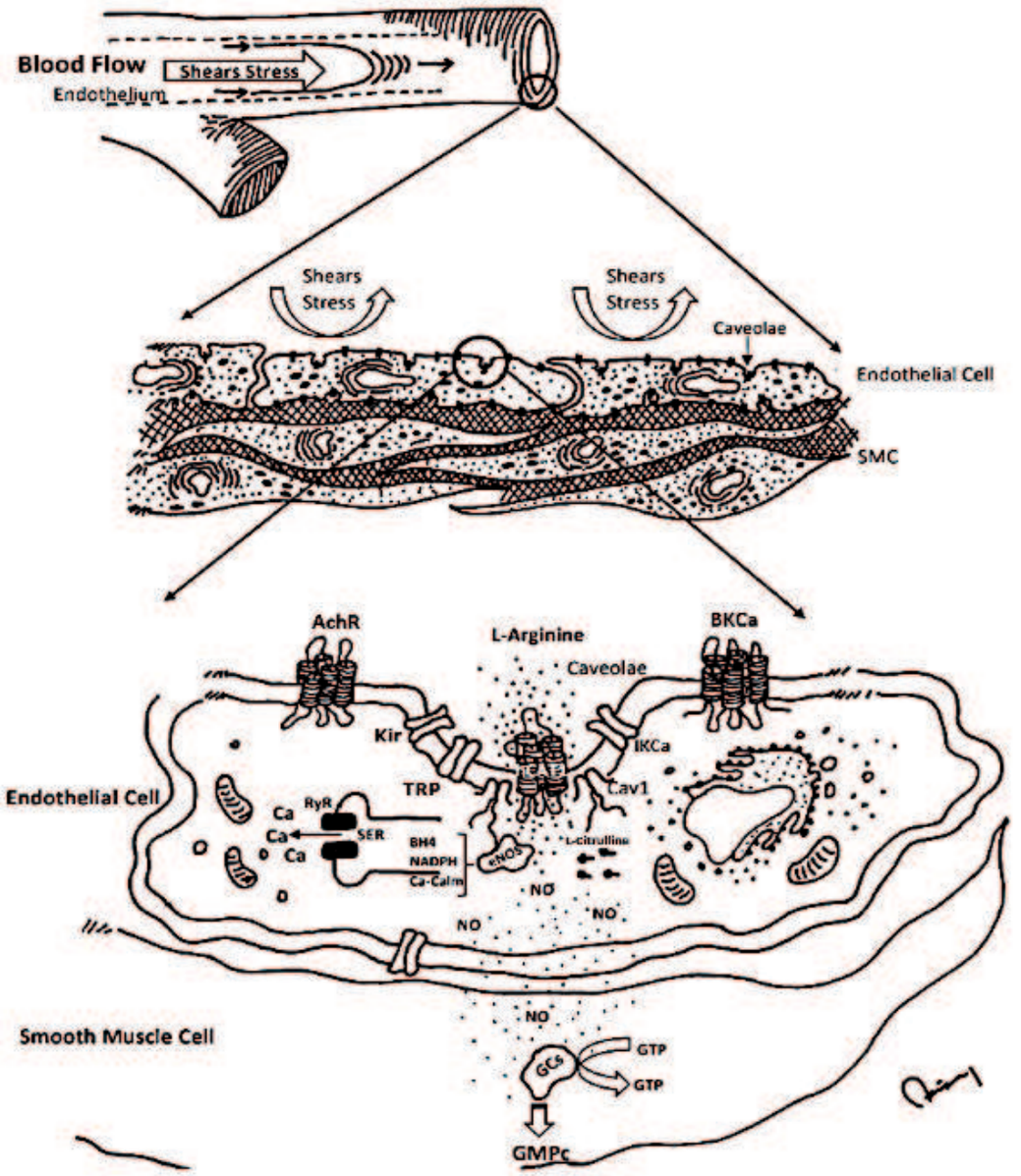


**Figure 5.**
*Role of endothelium in the regulation of vascular tone. Endothelial cells, as a part of blood vessels walls, respond to mechanical stress induced by flow (shear stress) by activation of L-arginine/NO pathway to induce the NO release and relaxation of smooth muscle cells (SMCs). Subcellular localization of hCAT-1 in caveolae is relevant for its function, and the role of potassium channels (BKCa, mainly) has been recently described as important for endothelial cells function. The activity of the endothelium is regulated by different agonists like acetylcholine (Ach) through plasma membrane receptor (AchR) and others like insulin or serotonin, etc.*

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