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Protein Kinase A and Protein Kinase C Connections: What Could Angiogenesis Tell Us?

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Abstract

The formation of embryonic blood vessels, defined as vasculogenesis, is a complex morphogenetic process ultimately related to tubulogenesis, carried out from *in situ* differentiation of mesoderm-recruited or proliferated progenitor endothelial cells (angioblasts) to endothelial cells for structuring a primary vascular plexus. Subsequent events involving apoptosis versus cell survival (remodeling) in the vessel network stabilizes the primordial microvasculature, which through the angiogenesis process yields new capillaries by sprouting from the preexisting ones. Methylxanthinic alkaloids such as caffeine (compounds present in a number of beverages consumed worldwide) exert some well-known effects upon heart and other cardiovascular structures, in part, by negatively interplaying with phosphodiesterase (PDEs) enzymes. Once caffeine as well as *Ilex paraguariensis* (yerba mate) infusion extract have shown to enhance the vessel formation (vasculogenesis and angiogenesis), we discuss the impact afforded by *I. paraguariensis* constituents on the (PDEs-related) quantities and stability of Protein kinase A (PKA) and Protein kinase C (PKC) enzymes. Besides, the text reflects on a suggested dual roles displayed by PKA and PKC enzymatic pathways in the developmental angiogenic events.

Keywords: Protein kinase A (PKA) and protein kinase C (PKC), Cyclases and phosphodiesterases, Methylxanthinic alkaloids, Vessels remodeling, Angiogenesis and vasculogenesis

1. Introduction

Angiogenesis and vasculogenesis are the better studied processes of vessel formation [1]. Angiogenesis starts from preexistent vasculature, these last structures being either the primitive vascular plexuses primordially formed by vasculogenesis in the embryo or the postcapillary venous compartment of the mature vascular systems [2, 3].

Vasculogenesis is defined as the formation of early embryonic blood vessels from *in situ* differentiation of mesoderm-recruited/proliferated progenitor endothelial cells (angioblasts) to endothelial cells [4, 5]. This process involves endothelial precursor cell clusters organization (blood islands), in the yolk sac membrane (YSM), laying down a primary vascular plexus [6–8]. A subsequent remodeling of this vascular network – a process that combines events of cell death or regression in some vessels and survival or enhancement in others – gives rise to a more refined and effective microvasculature [9–11].

Further proliferation of capillaries sprouting from preexisting vessels is referred to as angiogenesis [12], a process involving coordinated endothelial cell proliferation and migration as well as recovering of extracellular matrix (ECM), tubule formation (tubulogenesis), and expansion of the surrounding vascular tissues [13–15]. Despite angiogenesis in adults being a rare event, it plays a fundamental role in physiological processes, such as the reproductive cycle of fertile women and the wound healing process [16, 17].

There are evidences that the vasculogenesis process that works in the early embryo forming primary vessels at high rates to keep pace with the growth of the body has been adapted, under certain situations, in the adult [4, 18, 19], since bone marrow-derived endothelial progenitor cells in the peripheral blood of adult animals and humans have been shown to be incorporated into neovascularization [3, 20]. Under such conditions, cytokines can be produced to induce the formation of vascular networks alluding to vasculogenic mimicry [13, 21]. Thus, in accordance with this concept, the embryonic cellular mechanisms (proliferation and differentiation) underlying vasculogenesis process would be, in some level, recapitulated in adult life [21–23].

The cardiovascular system is susceptible to positive chronotropic and inotropic actions afforded by a class of compounds like xanthines which cause dilatation in a number of blood vessels (on lung and kidney, e.g.) and constriction in some others, such as the one occurring in brain vessels, revealing their controversial pharmacological features and biological targets diversity [24]. Methylxanthinic alkaloids, such as caffeine and theophylline are majoritarian compounds present in the coffee and cola beverages as well as in various tea extracts [25, 26]. Thus, in particular, caffeine may possibly be one of the most consumed substances all over the world. Its tropism on the cardiovascular structures and other organ systems is already reasonably known [27], as the specific-tissue mechanisms of action in some processes waits for further elucidation. Otherwise, methylxanthinic alkaloid interaction with protein kinase A (PKA) pathway has a remarkable effect on several vessel-related events. For example, Shafer et al. has verified that the treatment with caffeine and other methylxanthines increases cAMP level by inhibiting cAMP phosphodiesterase (PDE) [28]. As cAMP activates PKA, glycolysis is elevated which increases the amount of ATP available for muscle contraction and relaxation.

Caffeine, as well as *Ilex paraguariensis* St. Hill., Aquifoliaceae (e.g., mate) infusion extract (1.03–4.12 μM), have been shown to increase the microvessels number, due to the enhancement on vasculogenesis and angiogenesis rates, in the model of yolk sac and chorioallantoic membranes of chick embryos [29]. Moreover, an additional stimulant property on embryonic metabolism was evidenced by the increase in the body growth (defined by the body length). The pharmacological effects of caffeine and theophylline present in the mate drinks on the cardiovascular system are mainly addressed to PDEs inhibition, which directly impacts the quantity, stability, and cell activities of PKA and Protein kinase C (PKC) [30]. In fact, the relaxant effect in the smooth muscle is attributed to PDE inhibition, with the consequent increase in cyclic adenosine monophosphate (cAMP) concentration [27, 31]. Moreover, the heart muscle stimulation and the bronchial muscle relaxation are mediated by beta-adrenoceptors stimulation and adenylate cyclase (AC) stimulation. It is also suggested that the competitive antagonism exhibited by methylxanthines on the adenosine receptors (A_1 and A_2) determines some of its complex effects [32, 33].

The action mechanism of caffeine and mate extract/tea upon the processes of vessel formation remains unclear despite the important evidences of xanthine involvement-related biological targets (PDE–AC) on the cardiovascular physiology. Thus, it seems important to pay attention to the suggested dual roles of PKA and PKC enzymatic pathways in the angiogenesis.

2. Distinct roles of PKC and PKA in angiogenesis

PKC isoforms are key mediators in hormone, growth factor, and neurotransmitter-triggered pathways of cell activation [34]. Proteomic technologies (gel-based and gel-free analyses methods) and metabolomics have been successfully used in the study of protein kinases. The application of these novel tools and strategies in the field of kinase signaling has been focused on the role of PKC in the heart (for review, see [35]). Another recent review provides, with particular attention, information on the role of PKC isoforms in the cardiovascular complications [36]. A scheme of endothelial signaling pathways is displayed in Figure 1. As reported by Wright and co-workers, the DAG–PKC pathway activated by vascular endothelial growth factors (VEGFs) contributes to the vascular function in many ways, such as the regulation of endothelial permeability, vasoconstriction, extracellular matrix (ECM) synthesis/turnover, leukocyte adhesion, cytokine activation, cell growth, and ultimately, angiogenesis (Figure 1-1) [37]. In fact, such role of PKC on the angiogenesis activation was confirmed by *in vitro* and *in ovo* experiments.

An interesting study related with the PKA *versus* PKC actions on angiogenesis was performed by DeFouw and DeFouw [38]. These researchers showed that whereas the exogenous activation of cAMP by PKA pathway signaling acts decreases the macromolecules extravasation in the chick chorioallantoic membrane (CAM) during early angiogenesis (4.5-day CAM, i.e., 4.5 days of embryonic development; stage 24-HH) [39], the PKC activity contributes, at least in part, to CAM endothelial hyper permeability (a crucial pro-angiogenic event) at the 4.5-day chick embryo. Nevertheless, it was already reported [40] that the cyclooxygenase (COX-2)

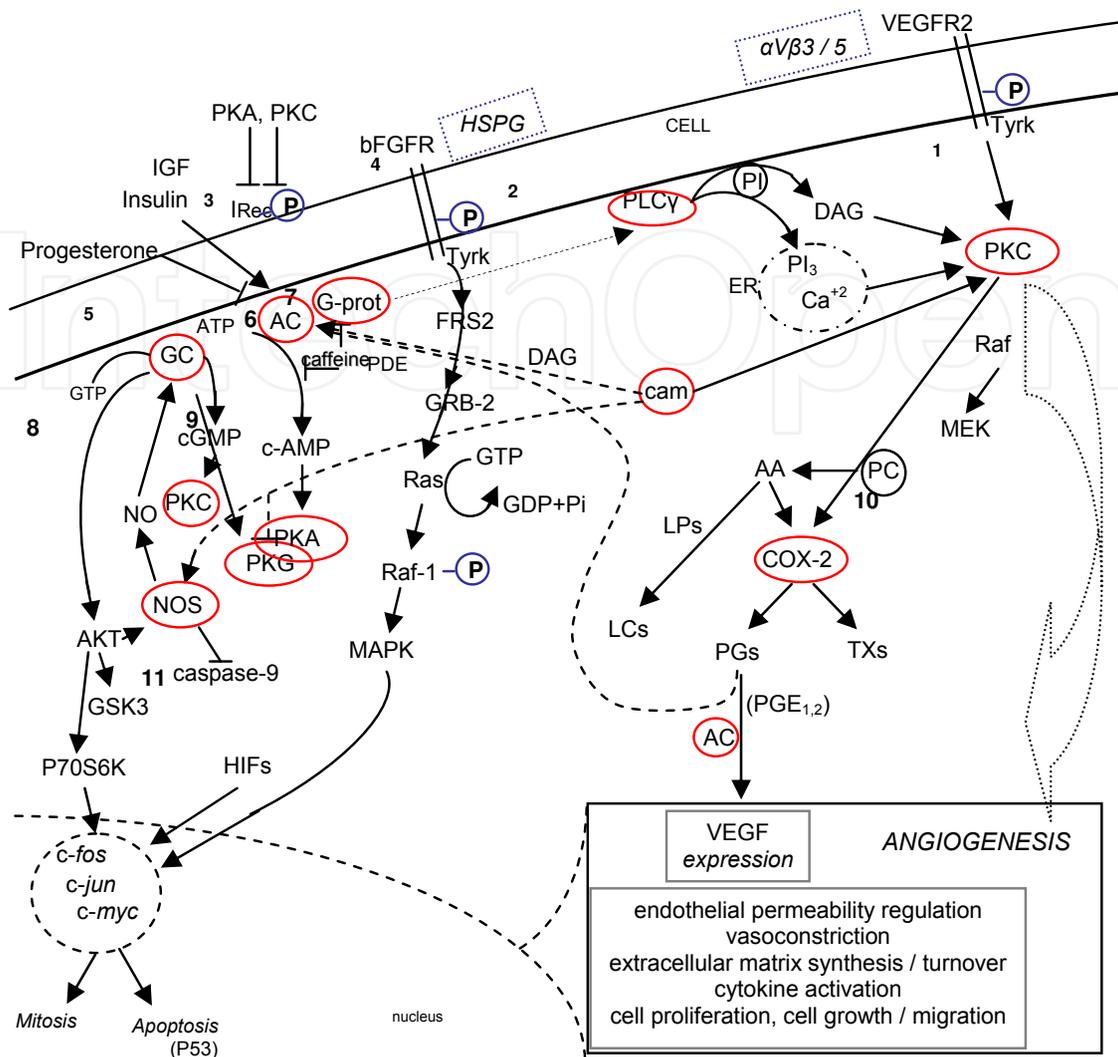


Figure 1. Schemas of endothelial signaling pathways. Basic fibroblast growth factor (bFGF) has been shown to activate a number of intracellular signaling pathways. Some well characterized processes that have been reported in endothelial cells and other cell types are shown. Many details in the steps of the processes were omitted for the sake of clearness and the numbers are included to enable the signals/effectors identification (then numbers not necessarily represent a sequence on transduction pathways, which are often non-exclusive). The autophosphorylation is activated by several tyrosine residues of FGFR and VEGFR. Some of the phosphotyrosine residues are binding sites for proteins with phosphotyrosine-binding domains such as FGF receptor substrate 2 (FRS2) that functions as docking protein and binds to the GRB2 which then can activate RAS. RAS may recruit RAF-1, a kinase whose action results in activation of a mitogen-activated protein kinases (MAPK) cascade. MAPK translocation to the nucleus proceeds activating transcription factors. PLC activation also plays a relevant role by causing the hydrolysis of phosphatidylinositol (PIP₂) to inositol-3-phosphate and diacylglycerol (DAG) leading to calcium release and activation of protein kinase C (PKC). These kinase/eicosanoid-mediated signal transduction pathways can lead to a number of biological responses on the cell housekeeping that involve cell proliferation, migration, and the other mechanisms related to the endothelial cell phenotype (1–4). Guanylyl cyclase (GC) mediated survival promotion by means of AKT-NOS activation (5–8) and guanine triphosphate/cyclic guanine monophosphate (GC-cGMP)-PKG, as well as PKG activation pathways (5–9). Phosphodiesterase (PDE) inactivation, as attained by xanthines (caffeine, for example), with the consequent up-regulation of cAMP-PKA signaling and the down-regulation of cGMP-PKG (6-7). A PDE compensatory role on the cAMP/PKA probable anti-proliferative (and/or anti-EC migration) effects afforded, as suggested, by a potent stimulus (from PIP₂, for example) on the PKC mitogenic pathway, with subsequent COX-2 activation (10), or also by pro-vascular signals transmission contributions (11).

pathway, as well the AC–PKA signaling, enhances angiogenesis *in vivo* through induction by VEGF. Other studies have also indicated PKA as a positive angiogenesis regulator [41–45]. In this sense, PKA inhibition with H89 (PKA inhibitor) blocks vasoactive intestinal peptide-induced VEGF production and inhibits brain vascular endothelial cells proliferation [41], while PKA stimulation via Forskolin increases angiogenesis through PKA-dependent VEGF expression [42]. Also, Zhang et al. have demonstrated that the proinflammatory prostaglandin E2 (PGE2) promotes angiogenesis through activation of endothelial cell-expressed EP4 and PKA catalytic γ subunits. Furthermore, suppressing the expression of PKA activated substrates (i.e., Rap1A, HSPB6, or endothelial NO synthase) inhibits the tube formation, while the knockdown of RhoA or glycogen synthase kinase 3 β , that are inactivated after PKA phosphorylation, increases the tube formation of human microvascular endothelial cells [43].

In opposition to the concept of PKA-activated angiogenic events, some evidences have established a profile of angiogenesis inhibition and an endothelial cell survival decrease mediated by PKA [46]. However, these authors have also demonstrated that basic fibroblast growth factor (bFGF)-stimulated blood vessel branch points were non-abolished by concomitant treatments with cAMP or PKA_{cat}. A subsequent study [47] demonstrated, in human granulosa cells, the PKA-mediated negative regulation of vessel formation (as well as the modulation of endothelial cell survival) related to the increase on mRNA levels of angiopoietin-2 (ANG-2; a pro-apoptotic agent) by both PKA and PKC activators (8-Cl-cAMP and ADMB), whereas the respective inhibitors (GÖ 6983 and Rp-cAMP) markedly decreased the levels of ANG-2 mRNA. Concurrently, VEGF-induced human umbilical vein endothelial cells (HUVECs) migration and proliferation were decreased by PDE2 and PDE4 inhibitors [48]. Additionally, Jin et al. have shown that PKA activation blocks pp60Src-dependent vascular endothelial–cadherin phosphorylation which stimulates cell–cell adhesion and inhibits endothelial cell polarization and migration, which consequently blocks sprouting in newly forming embryonic blood vessels [49]. In prostate tumor epithelial cells, the cAMP derivative 8-pCPT-2'-O-Me-cAMP, a weak agonist of PKA, acts via stimulation of that kinase that, in its turn, antagonizes Rap1 and hypoxic induction of 1 α protein expression, VEGF production and, ultimately, angiogenesis [50]. More recently, Liu et al. have proposed that the major PKA function in physiological condition may be to inhibit angiogenesis through REG γ –proteasome mediated regulation. It has been shown that REG γ interacts with protein kinase A catalytic subunit- α (PKA α reducing its intracellular stability) in HUVECs and mouse embryonic fibroblast cells (MEFs). The study has evidenced that REG γ antagonizes PKA pathway and facilitates VEGF-induced expression of pro-angiogenic genes (e.g., vascular cell adhesion molecule-1 gene [*VCAM-1*] and endothelial-Selectin gene [*E-Selectin*]) through PKA-FoxO1 pathway. Nevertheless, authors empathize that the role of PKA on angiogenesis can vary depending upon different cell context and various signal cascades in physiological or pathological environments [51]. The anti-angiogenic role of PKA through different mechanisms represents useful tools to inhibit pathologic angiogenesis. Taken in the whole, the above cited results show contrasting actions upon angiogenesis, not only between PKA and PKC actions, but also involving each enzymatic pathway, *per se*.

3. How can xanthines interplay with vascular mediators?

As referred earlier [29], the treatments performed by 1.03–4.12 μM caffeine and mate extract, besides increasing vasculogenesis and angiogenesis concomitantly, have promoted embryonic growth as featured by increase in body total length of treated 4-day chick embryos. These findings may be better understood taking into consideration the findings previously reported by Shibley and Pennington [52]. These researchers have demonstrated that non-acute *in vivo* treatment of cultured 5-day-old chick embryo cells with 1 μM phorbol ester leads to down-regulation (instead of up-regulation as afforded by acute treatments) of PKC activity, significantly increasing the insulin-dependent amino acid intake/uptake and transport that are crucial processes for embryonic growth.

On the other hand, PKC has also been shown to be involved in the regulation of glucose (a well-known angiogenic activator and fetal weight and length-increasing factor) transport in adipocytes [53] and that this transportation activity was blocked by PKC inhibition. Indeed, hyperglycemia (15 mM glucose), as well as VEGF, are able, via VEGFR-2, to up-regulate PlGF (placental growth factor; a member of the VEGF family), which also acts as a survival factor for microvascular endothelial cells by preventing apoptosis [54, 55]. These evidences are concurrent with a time-dependent diacylglycerol (DAG)-mediated PKC activation event (Figure 1-2) in response to insulin and insulin-like growth factors activation [56].

Even though the impairment on nutrient transport related to PKC inhibition has been already demonstrated by Christensen et al. [53], possible remarkable compensatory responses exerted, for example, by insulin-like growth factor interaction with AC on the body length of the caffeine-treated embryos should be considered (Figure 1-3) [27].

4. What about phosphodiesterases?

Bearing in mind that the evidences of vasculogenesis and angiogenesis inhibition are related to PKC/PKA pathways, one could yet ponder that those effects not necessarily point to PDE-related action or additional AC-cAMP inhibitors, as the progesterone hormone, for example. It is plausible to assume that caffeine and mate effects might, at least in part, involve other angiogenic pathways than AC-cAMP-PKA inhibition, such as those related to phosphatidylinositol-2-kinase (PI2K) and calcium/DAG-PKC activation, or its collateral induction by bFGF [57], which is a crucial angiogenic growth factor (Figure 1-4). Besides, the tumor necrosis factor- α (TNF- α) and/or the guanylyl cyclase-cyclic guanine monophosphate (GC-cGMP-PKC/PKG), pro-angiogenic activating pathways are also worth mentioning (Figure 1-5). Notwithstanding, the relevance of PDE involvement in vasculature development is evidenced by the concept which the differentiation of a restrictive angiogenic-endothelial barrier function *in vivo* would include inactivation of PDE III and PDE IV. This implies in up-regulation of cAMP-PKA signaling (Figure 1-6) and down-regulation on cGMP-PKG pathway [38]. Moreover, (1) PDE2, PDE3, PDE4, and PDE5 are expressed in HUVEC; (2) both EHNA (20 μM), a PDE2 selective inhibitor, and RP73401 (10 μM), a PDE4 selective inhibitor, are able to enhance the

cAMP intracellular levels in HUVECs; (3) EHNA and RP73401 are able to inhibit cell proliferation, mitotic cycle progression and migration on HUVECs stimulated by VEGF; (4) HUVEC treatments with the cAMP analogue 8-Br-cAMP (600 μ M) mimicry the cAMP *in vitro* inhibitory effects; and (5) only the association of EHNA and RP73401 (co-treatment by PDE2 and PDE4 selective inhibitors) blocks angiogenesis *in vivo*, indicating that to start antiangiogenic activity both migration and cell proliferation must be conjointly abolished [48].

In addition, the relevant study published by Netherton and Maurice [58] punctuates that human vascular endothelial cells (VECs) express variants of PDE2, PDE3, PDE4, and PDE5 families and demonstrate that the levels of these enzymes differ among VECs derived from aorta, umbilical vein, and micro vascular structures as those present in the yolk sac/chorioallantoic membrane (YSM/CAM) of chick embryos. As stated by those investigators, it is noteworthy that the selective inhibition of PDE2 does not only fail to increase cAMP in any VECs lineage, but also it did not inhibit migration in the VECs studied.

Otherwise, the inhibition of PDE4 activity decreased cell migration but, in association with forskolin (an AC/GC activator), increased cAMP in all VECs studied [58]. PDE3 inhibition potentiated forskolin-induced increases in cAMP and also inhibited migration in VECs derived from aorta and umbilical vein, but not on microvascular VECs. From these data, one should expect that methylxanthines had reduced vessel number in the early extra-embryonic membranes (YSM and CAM) in response to PDE inhibition (Figure 1-7), by antagonizing adenosine, or indeed by protecting cAMP from degradation. However, there are some evidences concerning the process of microvessels development where the opposite has just been found. The cAMP pathway truly “rivals” with the angiogenic microenvironment in complexity (for inhibiting inflammatory cytokines) and constitutes a kind of cross-junction to which converges a significant number of cell signaling ways. Then, during vessel formation, cAMP (and its distinct cellular roles) is surely under influence of factors as diverse as different time-space conditions, distinct main regulative pathways, and a number of second messengers/effectors in various signaling routs/cascades. Moreover, these events are dependent on each vascular endothelial cell lineage and the biological system or study model considered.

5. Focusing on the environment of developmental microvessels

Embryonic microvessels (such as those growing in the 4-day chick YSMs/CAMs) are structures physiologically under one primordial choice: that is potentially “life or death” [10]. Therefore, despite the proinflammatory cytokines blockade due to cAMP increase mediated by PDE inhibition in response to methylxanthines action, and also the presence of eventual apoptotic stimulus (such as insulin/IGFs-PKA interaction-mediated cell death), the embryonic endothelial cells may be concomitantly exposed to powerful survival stimuli, for example, vascular growth factors; survival factors (i.e., ANG-1), guanylyl cyclase (GC)-Akt (i.e., GC-PKB) [59] (Figure 1-8), pericyte-support; blood flow; and others. Besides, specific pro-angiogenic signals/conditions (NO-synthase/NO-GC, intermittent hypoxia, and GC-PKC, e.g.) would be preponderant to protect the ECs (Figure 1-9) [60, 61]. In the light of these evidences, it is still plausible

to suggest that both caffeine and the *I. paraguariensis* extract may exert a compensatory role on the cAMP/PKA probable anti-EC proliferative effect and/or anti-EC migration effect, by means of potent stimuli (from PIP₂, Ca²⁺, e.g.) to the PKC mitogenic pathway, with supplementary COX-2 prostaglandin-E (PGE_{1,2}) activation (Figure 1-10). Additionally, pro-vascular integrins/cytokines contributions and GC-Akt-P70SK-related *c-fos* and *c-jun* activation (Figure 1-11) should be considered. In the context of the dual effect between the AC-cAMP and GC-cGMP functions in the ECs (concerning the up-regulation of cAMP-PKA signaling against the down-regulation on cGMP-PKG pathway), it is possible to ponder on a non-improbable straightforward antagonist action of PKC on the PKA pathway. In fact, this idea is in part supported by evidences that PKC is able to phosphorylate also PKA-specific consensus sites of Tnl (troponin 1), a cardiac myofilament [62].

As an alternative hypothesis concerning a compensatory mechanism on angiogenesis, negative modulation by cAMP, we suggest the improvement of glucose (an angiogenic activator) uptake by ECs, possibly mediated by insulin/IGF-AC activation in response to methylxanthine administration. As support for this idea, data provided by Hashimoto et al. [63] have shown that inhibitors of PKA and PI3K completely attenuated the NO-induced *in vitro* endothelial tube formation (from human aortic endothelial cells). These findings strongly suggest that PKA (Figure 1-12) and PI3K might both be mediating the angiogenesis process.

6. Conclusion

In conclusion, we should not rescind from the importance of considering some apoptotic level *per se* on the endothelial cells lineages (*anoikis*) during the transition events from immature vasculature, yielded by vasculogenesis, to a more stable and sophisticated one attained by angiogenesis. In the context of angiogenic remodeling [64], some microvessels “have to die for others to survive” becoming stable/quiescent vascular structures [9]. Many “puzzle pieces” of kinases pathways appear to be, up to date, lacking. For example, how to begin solving the metabolome matter related to PKA *versus* PKC pathways in the angiogenesis? In accordance with Agnetti et al. [36], the “one protein at a time” approach is unlikely to provide a comprehensive picture of the cellular signaling due to the concerted action of “several molecular players at the same time.” Thus, the activities of both PKC and PKA should not be considered so mutually exclusive characters in the scenery of developmental microvessel formation. However, the remarkable evidences on phosphodiesterases as possible pivotal target molecules for the angiogenic effects of caffeine and *Ilex paraguariensis* extract strongly suggest an antagonistic role of the protein kinases A and C in the same events.

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