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Adrenomedullin and Angiotensin II in Rat Cerebellar Vermis: Reactive Oxygen Species Production

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Abstract

Adrenomedullin (AM) is a peptide involved in blood pressure regulation. AM exerts its effects through the activation of three receptors, the AM type 1 (AM1), type 2 (AM2) and calcitonin gene-related peptide 1 (CGRP1) receptors. AM triggers several signaling pathways such as adenylyl cyclase (AC), guanylyl cyclase (GC), extracellular signal-regulated kinases (ERK) and modulates reactive oxygen species (ROS) metabolism. In brain, AM and their receptors are expressed in several localized areas, including the cerebellum. AM has been reported as an antioxidant. Recent evidence suggests the presence of an adrenomedullinergic and angiotensinergic system of physiological relevance in cerebellum vermis. To establish the role of AM in the regulation of cerebellar ROS metabolism, it was assessed the effect of AM and angiotensin II (ANG II) on three antioxidant enzymes activity: catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD), and on thiobarbituric acid reactive substances (TBARS) production in rat cerebellar vermis. The findings demonstrated that in cerebellar vermis, AM decreased and ANG II increased CAT, GPx and SOD activity and TBARS production. Likewise, AM antagonized ANG II-induced increase antioxidant enzyme activity. AM(22-50) and CGRP(8-37) blunted AM-induced decrease of antioxidant enzymes activity and TBARS production indicating that these actions are mediated through AM and CGRP1 receptors. Further, PKA inhibitor (PKAi) blunted AM action whilst apocynin and chelerythrine reverted ANG II action, suggesting that AM antioxidant action is mediated through stimulation of protein kinase A (PKA) activity, while ANG II stimulation occurs through protein kinase C/nicotinamide adenine dinucleotide phosphate oxidase (PKC/NAD(P)H oxidase) pathway. These results support the role of AM in the regulation of cerebellar antioxidant enzymes activity and suggest a physiological role for AM in cerebellum.

INTRODUCTION

Adrenomedullin (AM) is a multifunctional regulatory peptide with 52-(human) or 50-(rat) amino acids, member of the calcitonin / calcitonin gene-related peptide (CGRP) superfamily [1] with vasodilatory, hypotensive and growth - regulatory properties [1]. AM binds to and activates at least two specific receptors, type 1 (AM1) and type 2 (AM2), formed from the co-expression of a calcitonin receptor-like receptor (CRLR) and receptor activity-modifying proteins (RAMPs) 2 or 3, respectively [1, 2]. Some AM effects are mediated through the activation of calcitonin gene – related peptide 1 receptor (CGRP1), composed of CRLR and RAMP1 [3] and are antagonized by specific CGRP1 antagonist, CGRP(8-37) [4]. AM1 receptor is highly selective for AM over CGRP and other peptides, while both AM and intermedin bind with high affinity to AM2 receptor [5]. AM1 receptor is more effectively blocked by h-AM(22-52) or r-AM(20-50), two AM receptor selective antagonists, than by CGRP(8-37). AM is produced in cells of many tissues and organs, including the adrenal medulla, endothelial and vascular smooth muscle cells, myocardium, kidney, spleen, lung and several brain areas [4, 6, 7]. Likewise, AM receptors and binding sites are found in several body regions [8]. The

CRLR mRNA and peptide are predominantly expressed in the lung, blood vessels, liver, midgut, rectum, urethra, adrenal cortex, uterus, coronary artery endothelial and smooth muscle cells. RAMP1 expression have been reported in fat, thymus, spleen, uterus, pancreas and bladder; while RAMP2 and RAMP3 is expressed in the lung, kidney, heart, liver, spleen, uterus, ovary and placenta [2, 7, 8]. It has been established that AM functions as a circulating hormone and local paracrine/autocrine mediator with multiple biological activities [4]. AM plays an important role in cardiovascular homeostasis regulation. In fact, infusion of AM in human and in several species of experimental animals causes a potent and long-lasting, dose – dependent, hypotensive effect [7]. The mechanism of vasodilator effect mainly involves endothelium-independent relaxation, via CGRP1 receptors, and elevation of cAMP levels. In addition, AM elicits endothelium-dependent vasorelaxation mediated by nitric oxide (NO) [9]. In fact, AM activates endothelial NO synthase (eNOS) through the elevation of intracellular calcium levels and/or the activation of phosphatidylinositol 3 kinase (PI3K) and protein kinase B/Akt [4, 10]. AM may also contributed to blood volume regulation through its natriuretic and diuretic actions in the kidney and its effects on central nervous system (CNS) control of thirst and salt appetite [7, 11]. AM and its receptors are also expressed in the CNS and are particularly localized to the autonomic nuclei, including nucleus tractus solitarii (NTS), lateral parabrachial nucleus (LPBN) and rostral ventrolateral medulla (RVLM) and many regions of the brain including the cerebral cortex, thalamus, hypothalamus, brainstem, medulla oblongata, midbrain, amygdala and cerebellum [4, 6, 11-13]. These findings suggest the existence of a central adrenomedullinergic system of physiological relevance, especially in the central regulation of water and salt metabolism and cardiovascular function.

Adrenomedullinergic System in Cerebellum Vermis

The evidence suggests the existence of a functional adrenomedullinergic system in cerebellum. All of the components necessary for the active peptide and its receptors biosynthesis reside within the tissue. In fact, AM immunoreactivity, AM binding sites and CRLR, RAMP1, RAMP2 and RAMP3 expression are detected in rat cerebellum [11, 14, 15]. AM immunoreactivity was localized in the lateral, interpositus and medial nuclei; also in the molecular layer, Purkinje cells and the granular cells of the cerebellar cortex [11]. In addition, the presence of RAMP1 and RAMP2 mRNA in Purkinje cells and RAMP3 mRNA in cerebellar granular cells has been shown [16]. Likewise, CRLR and RAMP1 were detected on the surface of the Purkinje cell bodies and in their processes [17]. AM activates several signaling pathways in cerebellar tissue. In effect, AM activates extracellular signal-regulated kinases (ERK) and increases cyclic adenosine monophosphate (cAMP) production probably through the activation of protein kinase A (PKA). AM also increases cyclic guanosine monophosphate (cGMP) production and NO accumulation. These effects are mediated through the activation of AM1 receptor, since AM specific receptor blocker, AM(22-52), blunted AM action. Meanwhile AM-induced increment in cAMP production is mediated through stimulation of AM2 and CGRP1 receptors [14, 15]. Recently, we demonstrated that hypertension dysregulates AM cerebellar system and this is accompanied by an alteration of several signaling pathways. Effectively, quantification of cerebellar AM, CRLR, RAMP1, RAMP2 and RAMP3 expression using western blot analysis, demonstrated an up-regulation of cerebellar CGRP1 (CRLR+RAMP1) and AM2 (CRLR+RAMP3) receptors, concomitantly with a down-regulation of AM and AM1 (CRLR+RAMP2) receptor [14, 15]. These changes were associated with functional alterations, since hypertension blunted AM-induced increase in cGMP, NO production and ERK1/2 phosphorylation. Furthermore, Figueira and Israel [14] demonstrated that microinjection of AM into the cerebellar vermis of hypertensive rats caused a powerful and significant hypotensive response, which was specific and dose-dependent. This hypotensive effect was shown only during hypertension since in normotensive rats, AM or saline administration into cerebellar vermis increased mean arterial pressure in similar magnitude [14]. These results provide functional evidence *in vivo* for the important role of cerebellar AM in the regulation of blood pressure.

Adrenomedullin and Reactive Oxigen Species

Oxidative stress and inflammation are two interrelated biological events implicated in the pathogenesis of many diseases. Reactive oxygen species (ROS) are noxious substances largely produced under oxidative stress. Various forms of cellular stress constitute primary signals that are transduced into the cytoplasm and ultimately alter the expression of specific genes in the cell nucleus. One such form of cellular stress is the production of oxygen free radicals or ROS [18]. It has been shown that fibroblasts, endothelial cells, and smooth muscle cells, produce ROS at relatively low levels in response to cellular "activation" signals; so these molecules act as second messengers to regulate signal transduction pathways that ultimately control gene expression and posttranslational modifications of proteins, and also are being involved in a variety of diseases including hypertension [18]. ROS can be considered as signaling molecules which couple extracellular and cellular information and signals to nuclear signals in order to increase the production and expression of proinflammatory products [18]. In fact, it has been postulated that ROS act as second messengers in the induction of numerous cellular processes as they may be involved in modulating specific redox-sensitive signal transduction pathways as nuclear factor-kappa B (NF-kB) [19] activator protein-1 (AP-1) and signal transducers and activators of transcription (STATs), which entails the transcription of genes encoding cytokines, growth factors and cell adhesion molecules [20]. ROS can affect multiple signal transduction pathways upstream of nuclear transcription factors, including modulation of Ca^{2+} signaling, protein kinase, and protein phosphatase pathways [17]. In effect, the evidence show that ROS are involved in intracellular signaling through activation of tyrosine kinases and non-tyrosine kinases receptors, by inhibiting tyrosine phosphatases, increasing regulatory kinases activity, including PKC and PKA [21] and regulating intracellular calcium homeostasis [22]. Also, ROS are involved in signaling pathways involving the activation of mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinases 1/2 (ERK1/2) or p42/p44, c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (p38 MAPK) [22, 23]. Thus, ROS may function as a physiolog-

ical regulator of gene expression by modulating specific redox-sensitive signal transduction pathways and transcriptional regulatory events [18].

AM is a pleiotropic peptide which regulates ROS metabolism and the signaling pathways activated by these molecules. AM plays a protective role against oxidative stress as an endogenous antioxidant *in vivo* [24], due to its capacity to attenuate ROS production mediated by nicotinamide adenine dinucleotide phosphate oxidase (NAD(P)H oxidase) stimulation [24, 25]. AM can suppress ROS generation in cultured mesangial cells and macrophages [26], a mutual counteracting effect, because ROS was able to increase vascular endothelial and smooth muscle cells AM production [27]. It was postulated that in mesangial cells, PKA pathway has a functional role in inhibiting the activation of NAD(P)H oxidase [28]. Moreover, AM deficiency results in higher levels of ROS with subsequent vascular damage [24]. In rat ventricle, increased oxidative stress caused by ischemia-reperfusion injury can be attenuated by AM-mediated inhibition of NAD(P)H oxidase via NO-cGMP signaling pathway, suggesting a cytoprotective role for endogenous AM against organs damage [29]. It was shown that in AM+/– mice, AM has the potential not only to decrease blood pressure, but also to protect organs from damage [24]. Effectively, heterozygous mice for AM show perivascular inflammation in coronary arteries with an increased systemic and local oxidative stress, which is reversed with AM supplementation. Moreover, AM+/– mice accumulate higher oxidative stress and insulin resistance in aging compared with wild-type (WT) mice [30]. Age-related accumulation of oxidative stress is involved in blood pressure regulation and insulin resistance in aged AM+/– mice, thus AM as an endogenous substance is capable to counteract oxidative stress-induced insulin resistance associated with aging [30].

In the CNS, specifically in the cerebellum, AM has an important role in the regulation of cerebellar ROS metabolism. Indeed, AM decreased basal antioxidant enzymes activity: catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD), suggesting that AM can reduce ROS production in the cerebellar vermis, and thereby decreasing the activation of signaling cascades activated by these molecules. This decrease in antioxidant enzyme activity in cerebellar vermis indicates that AM is capable of reducing ROS production, because antioxidant enzymes activity reduction was accompanied by a decrease in lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS) production [14, 15, 31]. This action was mediated via stimulation of both AM and CGRP1, receptors. Thus, in cerebellum AM exerts its effects through several signaling pathways such NO/cGMP, AC/cAMP, ERK and modulation of ROS metabolism, suggesting a role of AM in the regulation of cerebellar antioxidant enzymes activity and supporting the presence of a functional local adrenomedullinergic system of physiological relevance.

There is evidence supporting the notion of an altered AM antioxidant action in hypertension, since it was observed that in spontaneously hypertensive rats (SHR), AM was unable to reduce basal SOD, CAT and GPx activity, and lipid peroxidation. Therefore, hypertension tiggers a dysregulation in the AM antioxidant capacity. The absence of AM response on the basal activity of antioxidant enzymes in the cerebellar vermis of SHR rats could be due to a pattern change in AM receptors components expression previously reported in the cerebellar vermis during hypertension [31]. Furthermore, oral administration of the antihypertensive compound, valsartan, was able to reduce peripheral blood pressure and reversed changes in cerebellar expression of AM, its receptors components, antioxidant enzyme activity and TBARS production of hypertensive rats [14, 15, 31]; indicating that these alterations represent the primary abnormality leading to hypertension; therefore reversion with peripheral administration of an antihypertensive, may represents a strategy to compensate the primary abnormality in blood pressure control and opens the possibility of a novel mechanism in antihypertensive therapy.

Cerebellar Angiotensin II and Reactive Oxigen Species

Angiotensin II (ANG II) is considered the main effector of the renin angiotensin system (RAS) and its actions are mediated through the stimulation of two specific receptors, type 1 (AT1) and type 2 (AT2) [32]. In the CNS it has been described the presence of a local RAS, which is involved in the regulation of autonomic control, hormone secretion and other functions [33]. In fact, ANG II-like immunoreactivity occurs in a discrete pattern throughout the CNS having a predominant association with regions involved in fluid and electrolyte homeostasis, cardiovascular control and neuroendocrine regulation [33]. AT_1 receptors are discretely localized in brain areas as circumventricular organs, including the subfornical organ, vascular organ of the lamina terminalis (OVLT), median eminence, anterior pituitary and the area postrema. Specifically, cerebellum contains all components of the RAS [34]. In effect, angiotensinogen mRNA and its peptide were shown in glial cell populations of all layers of the rat cerebellum, particularly in Purkinje cells and granular cells layers and within the cerebellar nuclei. Moreover, ANG II immunoreactivity has been found in Purkinje, granule, basket and stellate cells, and was localized in nuclei, and in some cell types, such as endothelial and granule cells [34]. AT_2 receptor-immunoreactivity in the cerebellum was primarily associated with the Purkinje cells layer and the deep cerebellar nuclei [35]. In 2-week-old rat, high level of AT_2 receptors are present in the inferior olive and cerebellar peduncles, which are associated with the establishment of the olivo-cerebellar connections [36]. In effect, chemical lesions of the inferior olive reduces ANG II binding to AT_2 receptors, not only in the inferior olive, but also in the cerebellar cortex of young rats, suggesting that AT_2 receptors are produced by inferior olive and transported through climbing fibers to the molecular layer of the cerebellar cortex [36, 37]. Studies on the effect of ANG II on cerebellum are sparse, but some studies in an *in vitro* neuron differentiation preparation provided evidence for a specific role of AT_2 receptors [36]. ANG II causes depression of spontaneous firing of Purkinje cells [38] stimulates astrocyte growth, ERK1/2 phosphorylation and the phosphorylation of Src and proline – rich tyrosine kinase -2 (Pyk2) in astrocytes obtained from cerebellum [39]. Therefore, it is plausible to assure the existence of a functional RAS in cerebellum.

Evidence indicate that NAD(P)H oxidase is a key source of vascular ANG II-stimulated anion superoxide production in vascular tissues, and a variety of peripheral tissues. Number of ANG II actions are mediated through the generation of

ROS [40, 41]. In particular, ANG II-induced hypertrophy is inhibited by over-expression of CAT, or by inhibition of vascular NAD(P)H oxidase [41, 42]. It is well established that ROS are important intracellular messengers of many physiological and pathological effects of circulating ANG II, since they are blocked by apocynin, a NAD(P)H oxidase inhibitor [43] or by CAT over-expression [28, 41]. In addition, ANG II has been shown to stimulate the increase of both monocyte chemotactic protein 1 (MCP-1) and vascular cell adhesion protein 1 (VCAM-1) mRNA expression in rat aorta. In these experiments, the increased expression of both VCAM-1 and MCP-1 by ANG II could be blocked by NAD(P)H oxidase inhibitors and CAT, suggesting that this enzyme may be contributing to oxidative stress and regulation of vascular inflammatory genes via the generation of hydrogen peroxide [40]. Furthermore, ANG II loading induces insulin resistance together with an increased ROS level, as measured by the urinary excretion of isoprostane, a marker for overall oxidative stress. The increase in ROS induced by ANG II can activate various signaling pathways.

ROS is also involved in ANG II-mediated signaling in the CNS [44]. In fact, ANG II stimulated superoxide generation in primary CNS cell cultures was prevented by the AT_1 receptor antagonist losartan [44]. In cerebellum, acute stimulation with ANG II increased antioxidant enzymes activity; this suggests that ANG II increases ROS production as this effect was also associated with an increase of lipid peroxidation products [15]. ROS increments induced by ANG II can activate various signaling pathways at cerebellar vermis level. In fact, activation of ERK1/2 and subsequent genes expression in vascular smooth muscle cells induced by ANG II is inhibited by pretreatment with antioxidants [45], suggesting its redox-sensitive signaling.

Antagonism between Adrenomedullin and Angiotensin II in Cerebellar Vermis

AM exert counter-regulatory effects on ANG II- induced actions at various levels and locations, since AM potently blocked the ANG II-stimulated intracellular ROS generation from NAD(P)H oxidase and the subsequent redox-sensitive gene expression, among others intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1), via a cAMP/PKA-dependent mechanism in rat aorta endothelial cells [25]. In addition, in human aorta smooth muscle cells, AM inhibited in a dose-dependent manner, ANG II induced-contraction and proliferation; and a stimulatory effect on proliferation of quiescent cells. The cAMP/PKA pathway was involved in the AM inhibitory effect of ANG II-induced proliferation [46]. Furthermore, AM decreased ANG II-induced collagen deposition surrounding the coronary arteries, inhibiting myofibroblast differentiation and expressions of extracellular matrix-related genes in rats. In effect, AM through its potent antioxidant activity, inhibits ANG II-induced activation of redox-sensitive JNK and genes expression such as MCP-1 and Nox1. Similarly, Yoshimoto et al. [25] found that AM was able to reduce ANG II-induced increase in ROS production in rat aorta endothelial cells. This effect was dependent of receptors and cAMPc-PKA signaling pathway [25]. Thus, AM through its potent antioxidant activity, inhibits ANG II-induced activation of redox-sensitive JNK and genes expression such as MCP-1 and Nox1, suggesting that AM plays a protective role as an endogenous antioxidant in ANG II-induce vascular injury. Furthermore, in AM-deficient mice, ANG II stimulation in tissues involved in insulin resistance, liver and skeletal muscle were found to produce higher levels of ROS.

AM and ANG II antagonism is also described in cerebellum. In effect, AM was able to inhibit basal and ANG II-induced stimulatory effect on antioxidant enzymes activity [15]. AM-induced decreased antioxidant enzymes activity and TBARS production observed in cerebellar vermis is mediated by both receptor subtypes: CGRP1 and AM receptors, since AM effects were completely inhibited with CGRP(8-37) and AM(22-52) pretreatment, known as CGRP1 and AM receptor inhibitors, respectively [15]. Similarly, as occurs in rat vascular smooth muscle cells were the inhibitory effect of AM on ANG II-stimulated ROS generation is mediated by AM and CGRP1 receptors [28]. Several are the pathways involved in AM counter-regulatory effects on ANG II-induced actions on antioxidant enzymes activity in rat cerebellar vermis. One of them is the mediation of PKA, since pretreatment with PKI-(6-22) amide, a PKA inhibitor, was able to reverse AM inhibitory effect on the antioxidant enzymes basal activity [15], as it has been reported by other studies in several tissues [24, 25]. On the contrary, PKA mediation is not related with ANG II-induce enzyme activation since PKAi did not alter ANG II action on antioxidant enzymes activity. When activation of NAD(P)H oxidase was explored, it was demonstrated that pretreatment with apocynin, a NAD(P)H oxidase inhibitor, completely blocked ANG II stimulatory action on cerebellar antioxidant enzyme activity while it was ineffective on the inhibitory action of AM. This clearly demonstrates a role for NAD(P)H oxidase in cerebellar ANG II action [15]. Indeed, blockade of NAD(P)H oxidase with diphenyleneiodonium (DPI) effectively inhibits ANG II induced ROS production [47]. In the CNS, Chan et al. [47] demonstrated that ANG II injection into the RVLM increased phosphorylation of ERK 1/2, p38MAPK and ROS production. These effects were attenuated by the application of DPI into RVLM, antisense oligonucleotides that target p22phox or p47phox mRNA, or the SOD mimetic tempol. In addition, losartan blocked phosphorylation of serine residues of p47phox induced by ANG II in RVLM, and manifested a temporal profile that correlated positively with $O_2^{\cdot-}$ production induced by the octapeptide. Furthermore, DPI or apocynin administration into the RVLM or cerebral ventricles reduced pressor response induce by ANG II in rats [47]. These data indicate that redox signaling, NAD(P)H-oxidase-dependent production of superoxide are involved in ANG II-induced stimulatory actions in cerebellar neurons, which explains the increases of antioxidant enzyme activity such as CAT, SOD and GPx.

It is well established in peripheral tissues and in brain that NAD(P)H oxidase is regulated by PKC, an enzyme which is known to be activated by ANG II [22, 48]. In fact, pre-incubation of hypothalamic tissue with the PKC inhibitor, chelerythrine, completely attenuated ANG II-stimulated antioxidant enzyme activity of CAT, SOD and GPx *in vitro* [48]. The possible role of PKC, NAD(P)H oxidase and antioxidant enzyme activation in ANG II signaling in cerebellum was shown by Figueira and Israel [15] since they demonstrated that the PKC inhibitor, chelerythrine, completely blocked

ANG II-induced antioxidant enzyme activity of CAT, SOD and GPx *in vitro*. Accordingly, are the studies in which it was shown that the specific inhibitor of PKC-α, Go-6976, attenuates the increase in the firing rate of the hypothalamus and brainstem neurons induced by ANG II, reduces dipsogenic response to intracerebroventricular (ICV) administration of ANG II and inhibited the natriuretic and kaliuretic response produced by ICV-ANG II [49, 50]. These findings and those presented in rat cerebellar vermis, unequivocally demonstrate that the increase in the antioxidant enzymes activity induced by ANG II is mediated via PKC/NAD(P)H oxidase pathway. In Fig 1 the possible mechanism of antioxidant /oxidant action of AM/ANG II in cerebellar vermis is proposed.

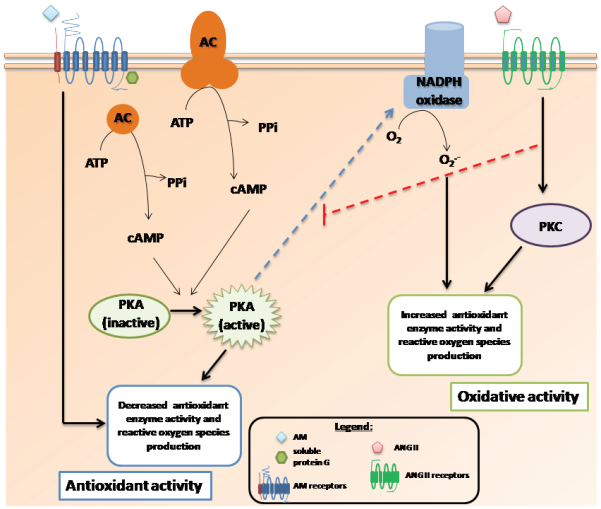

Figure 1: Proposed Mechanism of Action of AM in Rat Cerebellar Vermis. AM inhibits SOD, CAT and GPx activity through a PKA-dependent mechanism. Meanwhile, ANG II increases those enzymes activities via a PKC/NAD(P)H oxidase dependent pathway. AM antagonizes ANG II actions (With permission of Figueira and Israel, [14]).

It is reasonable to suggest that cross-talk between the AM receptor and angiotensin II receptor is responsible for the antagonistic effect between AM and ANG II on the antioxidant enzymes activity, possibly through the existence of a molecule which could intercept AM signaling pathway on the signaling activated by ANG II. Although indirectly, the results of Liu et al. [28] suggest that AM inhibits ANG II-induced oxidative stress via Src kinase- (Csk) mediated inhibition of Src activity. Effectively, in vascular smooth muscle cells, cAMP-PKA pathway is the main signal involved in AM-induced inhibition of ROS production, and PKA regulates Csk activity through its phosphorylation. Thus, Csk activation, via PKA activation, and inhibition of Src activation, could be this molecule candidate through which AM intersected ANG II stimulatory ROS signaling [28]. This assertion waits for further research in cerebellar tissue.

CONCLUSIONS

In conclusion, cerebellar AM is involved in the regulation of oxidative stress thus it can be inferred that this peptide plays an important functional role in this nervous system structure.

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CONFLICTS OF INTEREST

Conflicts of interest: none.

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AUTHOR S' CONTRIBUTIONS

Both authors, Figueira and Israel, participated in all aspects of the experiments, data processing, writing and edition.

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