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Research report

Candesartan prevents angiotensin II-induced facilitation of hypoxic neuronal damage through PKCδ inhibition

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Abstract

To investigate the role of protein kinase Cδ (PKCδ) in angiotensin II-induced facilitation mechanisms of hypoxic neuronal damage and whether candesartan, an AT1 receptor antagonist, can suppress these mechanisms, we performed in vitro experiments which were free from vascular components using PC12 cells under hypoxic (12 h)/reoxygenation (0–48 h) conditions. Angiotensin II apparently increased the basal expression level of PKCδ phosphorylated at Ser^{643} before hypoxia, promoted the cleavage of PKCδ to its catalytic fragment, and fostered the progression of DNA fragmentation after hypoxia. Candesartan inhibited both phosphorylation and cleavage of PKCδ and suppressed the angiotensin II-induced facilitation of DNA fragmentation under hypoxic/reoxygenation conditions. However, PD123319, an AT2 receptor antagonist, influenced neither PKCδ nor the angiotensin II-induced facilitation of DNA fragmentation. Furthermore, in PC12 cells expressing the ATP-binding mutant of PKCδ ($PKC\delta^{K376R}$) acting as a dominant-negative protein, both phosphorylation and cleavage of PKCδ were attenuated and DNA fragmentation was markedly suppressed regardless of the presence of angiotensin II. These findings suggest that angiotensin II-induced facilitation of DNA fragmentation under hypoxic conditions is mediated by PKCδ, and the mechanisms can be suppressed by the candesartan mediated blockade of the AT1 receptor.

Theme: Disorders of the nervous system
Topic: Ischemia

Keywords: Angiotensin II; AT1 receptor; DNA fragmentation; Hypoxia; PC12 cell; Protein kinase Cδ

1. Introduction

Angiotensin II (AngII), which is cleaved from angiotensinogen by renin and the angiotensin-converting enzyme (ACE), participates in the pathogenesis of hypertension [12,13,24,32]. The functions of two major AngII receptor subtypes, AT1 and AT2, have been studied using specific receptor antagonists such as candesartan and PD123319, respectively [8,12,13,24,32,34]. Growing evidence indicates that the AT1 receptor is responsible for the majority of the classical and known biological effects of AngII, including hypertension, vasoconstriction, and proliferation of vascular cells [21], and functions also as a significant contributor in the pathophysiology of ischemic stroke [12,13,24,32]. AT1 receptor stimulation results in cerebral vasoconstriction, thus contributing to the shift in cerebrovascular autoregulation toward elevated blood pressure [12,13,24,32]. AngII fosters pathological vascular remodeling [16] and increases expression of adhesion molecules leading to microvessel permeability and inflammation via AT1 receptors in endothelium of brain vessels [12,13]. Consistently, a high serum level of ACE can reportedly cause an increase in the volume of ischemic brain lesions [2].

There is emerging evidence indicating that antihypertensive therapy, which blocks the AngII system, is

Abbreviations: ACE, angiotensin-converting enzyme; AngII, angiotensin II; DMEM, Dulbecco's modified Eagle's medium; PC12, rat pheochromocytoma cells; PKCδ, protein kinase Cδ; TUNEL, terminal deoxynucleotidyl transferase (TdT) dUT nick-end labeling

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beneficial not only to lower blood pressure but also to reduce brain vulnerability to ischemia in hypertension [4,12,13,24,32]. AngII synthesis or AT1 receptor blockade improves neurological outcomes and reduces infarct volume in experimental cerebral ischemia [4,12,13,24,32]. Reduction in blood pressure alone does not seem to be sufficient for protecting against ischemia, as is evidenced by the failure of other types of antihypertensive drugs to reduce the size of ischemic infarcts [24]. The AT1 receptor blockade reportedly normalizes cerebrovascular autoregulation and reduces the loss in blood flow after middle cerebral artery occlusion in spontaneously hypertensive rats [13,24,32].

The beneficial effects of AT1 receptor blockade do not appear to depend exclusively on normalized cerebral blood flow [31]. Under conditions of middle cerebral artery occlusion, perfusion thresholds for ATP depletion are down-regulated in AT1 knockout mice [31]. Neurons from AT1 knockout mice exhibit a tolerance against oxygen-glucose deprivation in vitro [31]. These findings suggest a direct correlation between neuronal AT1 receptors and ischemic neuronal damage [31], although the underlying mechanisms remain to be elucidated. Naturally, under conditions of acute cerebral artery occlusion, neuronal death is unavoidable without recovery in cerebral blood flow. However, under conditions of transient or mild ischemia/hypoxia, neuronal AT1 receptor blockade may promote survival of neuron. The significance of PKCδ in signal transduction downstream to the AT1 receptor after binding with AngII was reported in vitro [8,23]. The purpose of the present study was to investigate whether PKCδ functions as a signal transducer downstream to the AT1 receptor after binding with AngII to promote hypoxic neuronal cell damage and whether the AT1 receptor blockade reduces neuronal cell damage by inhibiting PKCδ. PKCδ is a member of a large superfamily of isoforms that differ based on their requirement for lipid cofactors and Ca^{2+} for activation [3,18,19]. PKCδ, a Ca^{2+}-independent isoform, has been shown to regulate the mitochondrial-dependent pathway of apoptosis [5,9,20], which occurs in response to hypoxia/ischemia [7,33].

Because AT1 and AT2 receptors are expressed in cerebral arteries and microvessels as well as in neurons [22,25,28], it is difficult to clarify whether the effects of AngII are mediated by vascular or neuronal receptors by in vivo studies. To clearly define the involvement of neuronal AT1 receptors, we performed in vitro experiments under hypoxic/reoxygenation conditions using the rat pheochromocytoma cell line PC12 as well as candesartan and PD123319 as specific antagonists for the AT1 and AT2 receptors, respectively. Although PC 12 cells differ somewhat from mature neurons in the brain, PC12 cells more than 20 passages are known to express both AT1 and AT2 receptors [34] and considered as a usable model to study signal transduction system downstream to the neuronal AngII receptors.

2. Materials and methods

2.1. Preparation of cDNA and plasmids

Full-length murine PKCδ cDNA (GenBank, accession number: AF274044) was obtained from a murine brain cDNA library (TaKaRa Bio) by PCR using pfu polymerase (Promega). The mutagenesis reactions, in which the conserved lysine residue at amino acid 376 in the ATP-binding domain of murine PKCδ was changed to arginine [18], were performed by PCR using the PKCδ cDNA as a template, and the oligonucleotide 5′-AAGTACTTTGCAATCCGGTGTCTGAAGAAGGAC-3′ (underline: mutant sequence for the arginine substitution at amino acid 376) as one of the primers [18]. This mutant yields an enzymatically inactive *trans*-dominant protein [18]. The PCR products were separated electrophoretically, purified and treated with *Eco*RI, then inserted into the multiple cloning site of the pEF1 expression vector (N-terminal Xpress tagging vector) (Invitrogen) using the Rapid DNA Ligation Kit^{R} (Boehringer Mannheim). The transformed competent cells (Epicurian coli^{R} XL-2 blue: STRATAGENE) containing the ligation constructs (PKCδ^{K376R}pEF1 plasmid) were streaked onto LB-kanamycin-agar plates. The accuracy of PKCδ^{K376R}pEF1 plasmid construction was confirmed by restriction analysis and full sequencing of the inserted PKCδ^{K376R} cDNA using primer extension methods (TaKaRa Bio).

2.2. Cell culture and transfection

PC12 cells [11] (JCRB 0733; Human Science Research Resource Bank, Osaka, Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C without trypsin–EDTA for cell subculture. PC12 cells were transfected with the PKCδ^{K376R}pEF1 plasmid or pEF1 plasmid (without PKCδ^{K376R} cDNA; control) using Effectene™ Transfection Reagent (QIAGEN). At the first subculture, 48 h after transfection, culture medium was supplemented with 0.75 mg/ml G418 (Calbiochem) to positively select for stable integrants of PKCδ^{K376R}pEF1 or pEF1 (PKCδ^{K356R}pEF1 cells or pEF1 cells, respectively).

2.3. Induction of hypoxia

PC12 cells less than 20 passages are known to exclusively express the AT2 receptor and are used as a good model for studying AT2 receptor function [34]. However, the cells at more passage times express both AT1 and AT2 receptors [34]. Thus, we exposed PC12 cells, passaged between 25 and 30 times, to hypoxia. We used candesartan and PD123319 as specific antagonists for the AT1 and AT2 receptors, respectively. The following six