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Effect of Flavonoid Glycosides on Angiotensin II Induced Changes in Redox Status in PC12 Cells

Wpływ flawonoidów na zmiany stanu redoks indukowane angiotensyną II w komórkach PC12

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Abstract

Background. Angiotensin II (AngII) acts through two types of receptors: AT_1 and AT_2, whose stimulation results in mutually opposite effects. Contrary to AT_1 receptor, signaling pathway initiated by AT_2 receptor has been shown to activate various phosphatases. Cellular redox status plays a modulatory role in signal transduction, affecting kinases and phosphatases activity.
Objectives. The aim of this experiment was to investigate whether AngII through its AT_2 receptors is able to induce changes in redox status in PC12 cells. Besides, it was resolved to study the influence of luteolin-7-O-rutinoside and eriodictiol-7-O-rutinoside on cellular redox balance in PC12 cells with and without prior AngII stimulation.
Material and Methods. PC12 cells were preincubated with Losartan and either lutA or erioB for 10 minutes, followed by incubation with AngII for 0.5, 2 and 5 minutes. For comparison, PC12 cells were incubated with each of these substances alone. Subsequently, the cells were lysed and superoxide dismutase (SOD) activity was measured.
Results. After 2-minute AngII stimulation 3-fold SOD activity decrease was observed. This effect was abolished by lutA and erioB; preincubation with either of these compounds caused return of SOD activity to the values equal or slightly above the control level.
Conclusion. It might be suggested that AngII through its AT_2 receptor in PC12 cells induces decline in superoxide anion (SOD substrate) concentration followed by SOD activity decrease **(Adv Clin Exp Med 2004, 13, 6, 891–895)**.

Key words: angiotensin II, AT_2 receptor, PC12, flavonoid glycosides, superoxide dismutase.

Streszczenie

Wstęp. Angiotensyna II (AngII) działa przez dwa rodzaje receptorów AT_1 i AT_2, których stymulacja wywołuje przeciwstawne efekty. W przeciwieństwie do receptora AT_1, ścieżka sygnalizacyjna inicjowana przez receptor AT_2 aktywuje szereg fosfataz. Stan redoks komórki, wpływając na aktywność kinaz i fosfataz, może modulować przesyłanie sygnału.
Cel pracy. Celem eksperymentu było zbadanie, czy AngII przez receptor AT_2 może wpływać na równowagę redoks komórek PC12 oraz czy efekty wywołane przez hormon mogą być modyfikowane przez flawonoidy roślinne o właściwościach antyoksydacyjnych: 7-rutynozyd luteoliny (lutA) i 7-rutynozyd eriodykcjolu (erioB).
Materiał i metody. Komórki PC12 preinkubowano z Lozartanem i lutA albo erioB przez 10 minut, następnie z AngII przez 0,5, 2 i 5 minut. Dla porównania komórki inkubowano z lutA albo erioB bez stymulacji AngII. Po inkubacji komórki poddawano lizie i mierzono aktywność dysmutazy ponadtlenkowej (SOD).
Wyniki. Po 2-minutowej stymulacji AngII zaobserwowano 3-krotne obniżenie aktywności SOD. Ten efekt był zniesiony przez lutA i erioB; preinkubacja z obydwoma związkami powodowała powrót aktywności SOD do wartości porównywalnych z poziomem w kontroli.
Wniosek. Można przypuszczać, że AngII przez receptory AT_2 w komórkach PC12 indukuje obniżenie stężenia rodnika ponadtlenkowego (substratu SOD), co objawia się obniżeniem aktywności SOD **(Adv Clin Exp Med 2004, 13, 6, 891–895)**.

Słowa kluczowe: angiotensyna II, receptor AT_2, PC12, flawonoidy, dysmutaza ponadtlenkowa.

Angiotensin II (AngII) is a hormone maintaining cardiovascular homeostasis by controlling vascular tone, sodium excretion, hormone secretion and neuronal activity. It acts through 2 types of receptors belonging to the heptahelical receptors family: AT_1 and AT_2. Almost all of the known physiological effects of AngII are mediated through the abundantly expressed AT_1 receptors [1]. The AT_2 receptors are widely expressed in fetal tissues, but as development progresses, their expression is downregulated, and in adult animals it is restricted to the adrenal gland, brain, ovary, uterus, kidney, and heart [2]. A growing body of evidence suggests that there is a crosstalk between AT_1 and AT_2 in mediating the physiologic effects of AngII. Whereas stimulation of the AT_1 receptor leads to cellular growth and hypertrophy, angiogenesis, vasoconstriction, interstitial fibrosis and cardiac remodeling, AT_2 receptor stimulation causes opposite effects; antiproliferation and apoptosis, antiangiogenesis, vasodilation, decreased neointimal formation and inhibition of cardiac remodeling [3]. The AT_2 receptors have distinctive physiological actions in neuronal cells. They have been shown to inhibit calcium channels, activate potassium channels, leading to increased cellular polarization, and promote neuronal differentiation, including neurite outgrowth and upregulation of polymerized tubulin [4]. The signal transduction pathway triggered by the AT_2 receptor induction is not well defined. Nevertheless, its stimulation has been shown to activate various types of phosphatases such as MAP kinase phosphatase-1 and SHP-1 tyrosine phosphatase [4].

Numerous reports have shown that cellular redox status plays an important role in signal transduction pathways based on tyrosine phosphorylation/dephosphorylation processes. Redox agents, including reactive oxygen species (ROS), show modulatory effects on protein tyrosine kinases (PTKs) and phosphatases (PTPs) activity [5–7]. Superoxide and hydrogen peroxide (H_2O_2) once only known for their toxicity have now been recognized as signaling molecules [8, 9]. For example, in vascular cells AngII through its AT_1 receptors has been shown to stimulate NAD(P)H oxidase to produce superoxide anion, which is in turn converted to hydrogen peroxide by superoxide dismutase (SOD). Superoxide anion and hydrogen peroxide mediate AngII activation of various kinases (JnK, p38MAPK, ERK1/2) [3].

Factors capable of affecting redox balance are flavonoid glycosides present in many food products, that show numerous beneficial properties (for example are used in prophylaxis of atherosclerosis and prevention of neoplastic diseases) [10]. Flavonoid glycosides obtained from Mentha piperita: luteolin-7-O-rutinoside and eriodictiol-7-O-rutinoside have antioxidative and anti-inflammatory properties; the first compound has been shown to exert antiallergic effect (inhibiting histamine release) [11], and the second one (isolated from lemon) has revealed protective properties against oxidative damages caused by acute exercise-induced oxidative stress [12].

The aim of this experiment was to investigate whether AngII through its AT_2 receptors is able to induce changes in redox status in PC12 cells. Moreover, the influence of luteolin-7-O-rutinoside and eriodictiol-7-O-rutinoside on cellular redox balance in PC12 cells was studied. SOD activity determination was used as an indicator of redox changes.

PC12 cell line (rat pheochromocytoma cell line) was chosen for this study because of its expression of AT_2 receptors and sensitivity to AngII. Therefore, it makes an eligible experimental model to investigate signaling pathway triggered by AngII through AT_2 receptor stimulation.

Material and Methods

Material

Flavonoid glycosides; luteolin-7-O-rutinoside (lutA) and eriodictiol-7-O-rutinoside (erioB) were prepared from Mentha piperita at the Department of Pharmacognosy at Wroclaw Medical University. Superoxide dismutase kit was purchased from RANDOX Laboratories Ltd. (UK). Cell culture medium, angiotensin II, Bradford reagent, and chemicals for buffer preparations were from Sigma-Aldrich, Inc. (USA).

Experimental Scheme

The rat pheochromocytoma cell line PC12 was grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% horse serum and with 5% FBS (fetal bovine serum) at 37°C under humidified atmosphere of 95% air and 5% CO_2. Medium was changed every 48 h. Subsequently, PC12 cells were centrifuged, suspended in PBS buffer containing 0.01% glucose and 0.25 mM Losartan (2-butyl-4-chloro-1-[*p-(o-1H*-tetrazol-5-ylphenyl)benzyl]imidazole-5-methanol monopotassium salt), divided into samples, and incubated at 37°C. The experiment started with the addition of either luteolin-7-O-rutinoside (lutA) or eriodictiol-7-O-rutinoside (erioB) in 0.05 mM concentration for 10-minute preincubation, followed by incubation with AngII (10^{-7} M) for 0.5,

2, and 5 minutes. Samples incubated with lutA or erioB alone (for 10.5, 12 and 15 minutes) were treated as a reference. For comparison, one sample was incubated only with AngII (10^{-7} M) for 0.5, 2 and 5 min, and one was incubated at 37°C for 10.5, 12 and 15 minutes with neither AngII nor flavonoid glycosides (unstimulated PC12 cells). The experiment was ended with rapid cooling of the samples to 0°C and subsequent freezing them at –80°C. Eventually, the cells were lysed and superoxide dismutase (SOD) activity was measured, using RANDOX kit protocol.

Protein concentration was assessed due to the Bradford reagent protocol, using bovine serum albumin (BSA) as a standard.

Statistical Analysis

All of the results come from three independent experiments and the data are expressed as the mean. The level of significance was assessed with Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results

To make certain that AngII exerts its effects on PC12 cells through AT_2 receptor, Losartan was added to all of the samples to block AT_1 receptor.

Superoxide dismutase activity (SOD) (Fig. 1) decreased 3.3-fold after 2-minute AngII stimulation in comparison with 0.5-minute stimulation. After 5-minute stimulation SOD activity returned to the initial level. 10-minute preincubation of PC12 cells with erioB prior to AngII addition caused 5.1-fold increase in SOD activity after 2-minute AngII stimulation as compared to PC12 cells with AngII alone. Similar effect was observed in PC12 cells preincubated with lutA, where 4.1-fold increase in SOD activity after 2-minute AngII stimulation was noticed. It could be concluded that both compounds abolished AngII induced decrease in SOD activity. All other differences were statistically insignificant.

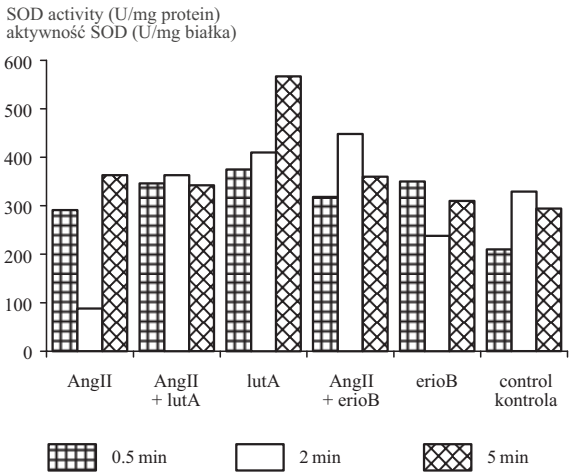

Fig. 1. Superoxide dismutase (SOD) activity in PC12 cells incubated with AngII for 0.5, 2, and 5 minutes (AngII); preincubated with luteolin-7-O-rutinoside for 10 minutes and incubated with AngII for 0.5, 2, and 5 minutes (AngII + lutA); incubated with luteolin-7-O-rutinoside for 10.5, 12 and 15 minutes (lutA); preincubated with eriodictiol-7-O-rutinoside for 10 minutes and incubated with AngII for 0.5, 2, and 5 minutes (AngII + erioB); incubated with eriodictiol-7-O-rutinoside for 10.5, 12 and 15 minutes (erioB); unstimulated PC12 cells (control)

Ryc. 1. Aktywność dysmutazy ponadtlenkowej (SOD) w komórkach PC12 inkubowanych z angiotensyną II przez 0,5, 2,0 i 5,0 minut (AngII); preinkubowanych z 7-rutynozydem luteoliny przez 10 minut i inkubowanych z angiotensyną II przez 0,5, 2,0 i 5,0 minut (AngII + lutA); inkubowanych z 7-rutynozydem luteoliny przez 10,5, 12 i 15 minut (lutA); preinkubowanych z 7-rutynozydem eriodykcjolu przez 10 minut i inkubowanych z angiotensyną II przez 0,5, 2,0 i 5,0 minut (AngII + erioB); inkubowanych z 7-rutynozydem eriodykcjolu przez 10,5, 12 i 15 minut (erioB); niestymulowane komórki PC12 (kontrola)

Discussion

Decrease in superoxide dismutase (SOD) activity after 2-minute AngII stimulation could result from decrease in superoxide anion concentration (SOD substrate). It might be suggested that AT_2 receptor stimulation triggers the pathway leading to reactive oxygen species (ROS) decrease. Such conclusion could be drawn from the emerging picture of AT_1 and AT_2 receptors as exerting opposed physiological functions. Whereas AT_1 stimulation induces phosphorylation processes resulting in MAP kinases activation [3], AT_2 receptor induces dephosphorylation reactions [13]. It has been shown, for example, that AT_1 induces ERK activation [3], and AT_2 – ERK inhibition [14]. Since in several cell types, AT_1 induction is known to stimulate ROS production, which in turn activates kinases, it could be postulated that AT_2, acting in an opposed way, would inhibit ROS production. Such a phenomenon has actually been observed in endothelial cells, where AT_2 receptor induction attenuated AT_1 receptor mediated superoxide formation by NAD(P)H oxidase [15]. The possible mechanism of AT_2 induced ROS inhibition could be based on the activation of nitric oxide synthase (NOS) and production of nitric oxide (NO). NO would then react with superoxide

anion, producing peroxynitrite anion $ONOO^-$, which would lower superoxide level and thus downregulate SOD. Besides, peroxynitrite has been shown to nitrosylate tyrosine residues on superoxide dismutase molecule, which could affect enzymatic activity [16]. Stimulation of nitric oxide formation by AngII through AT_2 receptors has been postulated in several studies [3, 4, 17, 18]. However, from many experiments it could be concluded that AngII stimulated increase in NO concentration requires bradykinin mediation. Such a hypothetical signaling pathway has been proposed for vascular cells, where AngII is thought to induce AT_2 receptors on vascular smooth muscle cells causing intracellular acidification and kininogenase activation, which is followed by bradykinin release and stimulation of its BK_2 receptors on endothelial cells. Finally, nitric oxide is released by endothelial cells and after penetration of vascular smooth muscle cells it activates cGMP resulting in vasodilation [19]. What mechanism could possibly account for nitric oxide generation in PC12 cells upon AT_2 receptor stimulation remains to be established.

Abolishing of AngII stimulated decline in SOD activity by pretreatment with lutA or erioB is confusing and difficult to explain. Assuming that AngII through AT_2 receptors actually stimulates NO synthesis, it is conceivable that both compounds interact preferentially with nitric oxide, thus preventing superoxide anion from being scavenged which would maintain stable SOD activity.

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