Effect of Epilobium angustifolium and Serenoa repens extracts on regulation of non-genomic signaling pathway of kinases

Wpływ ekstraktów z Epilobium angustifolium i Serenoa repens na regulację niegenomowego szlaku sygnałowego układu kinaz

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Abstract

Objectives: *Changes of kinase activity of non-genomic cellular signaling pathway may influence the effectiveness of pharmacotherapy in case of hormone-dependent tumors. Our study investigated a possible interaction at the molecular level between an aqueous herbal extract of Epilobium angustifolium as well as a lipid-sterolic fruit extract of Serenoa repens and synthetic drugs used in the treatment of hormone-dependent cancers.*

Material and methods: *E. angustifolium and Serenoa repens extracts were orally administered to testosterone-induced rats for 21 days. Changes of RafA/Mapk3/Mapk1 mRNA levels were analyzed by real-time quantitative PCR using target specific primers.*

Results: *The level of RafA mRNA slightly increased in rats receiving Epilobium angustifolium ($p=0.076$) and Serenoa repens ($p=0.016$) extracts. Administration of these extracts resulted in significantly elevated Mapk1 and Mapk3 transcripts in the investigated animals ($p<0.05$ for each extract). The levels of Mapk1 and Mapk3 mRNA strongly increased ($p<0.05$ for each extract) in animals receiving concomitantly testosterone and the extracts, while RafA transcription slightly decreased ($p<0.05$), as compared to controls.*

Conclusions: *The results of our study may indicate a potential effect of S. repens and E angustifolium extracts on the functioning of non-genomic cellular signaling kinases pathway. We investigated safety of these extracts to detect possible drug interactions between synthetic drugs used in the treatment of proliferative changes in hormone-dependent reproductive organs and herbal preparations.*

Key words: **menopause** / **rats** / **Serenoa repens** / **Epilobium angustifolium** / / **MAP kinases** / **non-genomic cellular signaling pathway** /

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Streszczenie

Cel: *Zmiana aktywności kinaz niegenomowego szlaku sygnalizacji komórkowej może wpływać na skuteczność farmakoterapii nowotworów hormono-zależnych. W badaniu analizowano na poziomie molekularnym wpływ wyciągu wodnego z liści ziela Epilobium angustifolium i lipidowo-sterolowego wyciągu z owoców Serenoa repens na możliwość wystąpienia interakcji z lekami syntetycznymi stosowanymi w terapii nowotworów hormono-zależnych.*

Materiał i metody: *Ekstrakty z Epilobium angustifolium i Serenoa repens podawano p.o. indukowanym testosteronem szczurom przez 21 dni. Zmiany poziomów mRNA kinaz RafA/MAPK3/MAPK1 analizowano metodą ilościowego PCR w czasie rzeczywistym stosując specyficzne startery.*

Wyniki: *U szczurów otrzymujących wyciąg z Epilobium angustifolium (p=0.016) i Serenoa repens (p=0.016) poziom transkryptu RafA uległ nieznacznemu podwyższeniu. Nastąpiła znaczna indukcja transkrypcji mRNA kinaz MAPK1 i MAPK3 u zwierząt pod wpływem badanych ekstraktów (p<0.05 dla każdego z wyciągów). U zwierząt otrzymujących jednocześnie testosteron i badane wyciągi poziomy mRNA Mapk1 i Mapk3 uległy podwyższeniu (p<0.05 dla każdego z wyciągów), podczas gdy ilość transkryptów RafA uległa nieznacznie obniżeniu (p<0.05), w porównaniu do grupy kontrolnej.*

Wnioski: *Wyniki badania mogą wskazywać na potencjalny wpływ wyciągów z S. repens oraz E. angustifolium na funkcjonowanie niegenomowego szlaku sygnałowego układu kinaz. W pracy odniesiono się do aspektu bezpieczeństwa stosowania badanych ekstraktów roślinnych w kontekście występowania ewentualnych interakcji pomiędzy lekami syntetycznymi stosowanymi w terapii zmian rozrostowych w hormono-zależnych narządach rozrodczych a preparatami ziołowymi.*

Słowa kluczowe: **menopauza** / **szczury** / **Serena repens** / **Epilobium angustifolium** / / **kinazy MAP** / **niegenomowy szlak sygnatomy układu kinaz** /

Introduction

Experimental observations indicate that estradiol, progesterone, and androgens could induce cellular and phenotypic effects evidencing the existence of the so-called non-genomic cellular signaling pathway, which is characterized by rapid action, and lack of involvement of transcription and translation processes from hormone-responsive genes [1-4].

A number of protein kinases are involved in this signaling cascade, including kinases belonging to the *Ras*/*RafA/MAPK* (rat sarcoma oncogene/rapidly accelerated fibrosarcoma protein/ mitogen activated protein kinases) axis [1-3].

A partial, tissue-specific deficiency of estrogen, which could be supplemented by activation of androgen aromatization reactions initiated by non-genomic *Ras*/*RafA*/*MAPK* signaling cascade, can be observed during menopause [4-7]. According to these observations, aromatase inhibitors and selective estrogen receptor modulators (SERMs) are often used in pharmacotherapy of hormone-dependent breast cancer [8-10]. We followed the assumption that administration of several herbal remedies, for example *Serenoa repens* and *Epilobium* sp. representatives, could elevate activation of mitogen activated protein kinases, inducing also androgen (testosterone) aromatization [11, 13-15].

In our study we aimed to prove the hypothesis that concomitant administration of certain herbal extracts with aromatase inhibitors and/or selective estrogen receptor modulators could result in an interaction potentially disruptive to the effectiveness of breast cancer therapy.

Material and methods

Plant extract

In this study, a commercial, standardized lipid-sterolic extract of the fruits of *Serenoa repens* was used (Fitoprost, Poland). Standardized dried aqueous extract of the herb of *Epilobium angustifolium* (0.91% m/m flavone glycoside compounds expressed as quercetin, 24.36% m/m phenolic compounds expressed as gallic acid, 0.09% m/m sterol compounds expressed as ß-sitosterol and 0.01% m/m tannin compounds expressed as pyrogallol) was obtained from the Institute of Natural Fibers and Medicinal Plants (Poznan).

Animal treatment

All procedures involving rats were performed in accordance with the Polish government regulations (01.21.2005, published in Dziennik Ustaw, No. 33; item 289), and in agreement with the Local Ethics Committee of the care and Use of Laboratory Animals in Poznan (No. 54/2007). Adult male Wistar rats (180–250 g, 4-weeks old) were kept at the Department of Pharmacology, Poznan University of Medical Sciences, in a climate-controlled room with 12h-light/dark cycle in plastic cages and allowed access to a commercial rat chow and tap water ad libitum. The animals were acclimatized for at least a few days before the experiment, and then they were castrated. All rats were randomly divided into 6 groups (10 animals each). All substances were administrated for 21 consecutive days.

One group of animals was administrated a lipid-sterolic extract of fruits of *Serenoa repens* [100 mg/kg/day, p.o.]. The second one was given dried aqueous extract of *Epilobium angustifolium* [100 mg/kg/day, p.o.]. The following 3 groups of rats were testosterone injected in order to induce a prostate enlargement. Induced control rats received testosterone [Testosteronum prolongatum; 100 mg/ml, Jelfa; 40mg/kg/day, 3 times, every 7 days; s.c.] dissolved in arachidonic oil [16]. Another group represented a combined treatment of rats with testosterone [100 mg/ml, Jelfa; 40mg/kg/day, 3 times, every 7 days; s.c.) dissolved in arachidonic oil and extract of *S. repens* [100 mg/kg/day, p.o.]). The last group of animals received concomitantly testosterone (in the above mentioned dose) and *E.*

angustifolium extract [100 mg/kg/day, p.o.]. Control rats were fed with arachidonic oil with H2O and PEG400 [50mg/kg/day; p.o.]. Sixteen hours after the last administration, rats were decapitated and prostate ventral lobes were immediately weighted, frozen in liquid nitrogen and stored at –80ºC until used.

RNA isolation and reverse transcription reaction

Total RNA isolation was carried out using TriPure Isolation Reagent (Roche Applied Science, Germany) according to the manufacturer's protocol. The integrity of RNA was visually assessed by conventional agarose gel electrophoresis and the concentration was tested by measuring the absorbance at 260 and 280 nm in a spectrophotometer (BioPhotometer Eppendorf). RNA samples were stored at –80°C until used. The 1 µg of total RNA from all samples was reverse-transcribed into cDNA using Transcriptor cDNA First Strand Synthesis Kit (Roche, Germany) and oligo(dT) 20 primer (Roche, Germany) according to the manufacturer's protocol. The obtained cDNA samples were stored at –20°C or used directly for the quantitative real-time PCR (qRT-PCR) reaction.

Real-time PCR assay

Gene expression level was analyzed by real-time quantitative PCR reaction using a LightCycler TM Instrument (Roche, Germany) and a Light-Cycler Fast Start DNA Master SYBR Green I kit (Roche, Germany) according to the instructions of the manufacturer. All primer sequences were designed using the Oligo 6.0 software (National Biosciences, USA), based on the sequence entries in the Genbank and synthesized from TIB Molbiol (TIB Molbiol Sp. z o.o., Poland). Sequences of primers for *RafA* and *Mapk3/Mapk1* genes (rapidly accelerated fibrosarcoma protein/ mitogen activated protein kinases) (rats homologues of human genes *RafA*/*ERK1/ERK2*) were designed using the Oligo 6.0 software (National Biosciences, USA), based on the sequence entries in the Genbank and synthesized from TIB Molbiol (TIB Molbiol Sp. z o.o., Poland), were as follows:

RafA-F: 5'- TTTTGGTTTGGCAACAGTGA -3';
RafA-R: 5'- TGATGTGGGAGTAGGGAAGC -3';
Mapk1-F: 5- AACCATTGAGCAGATGAAAG;
Mapk1-R: 5- GGTGCAGAACATTAGCTGA;
Mapk3-F: 5- ACGACCACACTGGCTTTCTT;
Mapk3-R: 5- GATTTGGTGTAGCCCTTGGA.

Thermal cycling conditions for nuclear receptor were as follows: for RafA, 40 cycles of 95°C for 8 s, 56°C for 7 s, 72°C for 8 s; Mapk1, 45 cycles of 95ºC for 8 s, 57 ºC for 7 s, 72ºC for 7 s; Mapk3, 40 cycles of 95ºC for 8 s, 58ºC for 5 s, 72ºC for 8 s. Primer specificity was verified by assessing s single PCR product on agarose gel and single temperature dissociation peak (melting curve analysis) of glyceraldehyde-phosphate-dehydrogenase (*GAPDH*) was used as the reference. All reactions were repeated twice. The data were evaluated using LightCycler Run 5.32 software (Roche, Germany).

Statistical analysis

The results were expressed as mean±SEM. Statistical significance of the differences between the control and study group was assessed by SPSS 17.0 (IBM Corporation, USA) software using one-way ANOVA test (and Fischer LSD post-hoc test). The values of $p<0.05$ were considered as statistically significant.

Results

Effect of *Epilobium angustifolium* extract on *RafA, MAPK1,* and *MAPK3* transcription profiles

Differences in the levels of the studied mRNA in each of the analyzed groups are presented in Table 1 and Figure 1.

Rats receiving the extract of *Epilobium angustifolium* had an increased *RafA* mRNA level by 114.4% ($p=0.076$). The levels of *Mapk1* and *Mapk3* transcript were also increased, by over 540% and 464.5%, respectively ($p<0.05$). The largest elevation of *RafA* mRNA ($p=0.124$) and *Mapk1* and *Mapk3* mRNAs (($p<0.05$) by 402.9%, 1404% and 1851.4%, respectively), was observed in testosterone-induced rats, as compared to controls ($p<0.05$). In animals receiving concomitantly testosterone and *E. angustifolium* extract, the level of studied *Mapk1* and *Mapk3* mRNAs increased by 51.6% and 20%, respectively ($p<0.05$), while the *RafA* transcription slightly decreased (by 13.1%) ($p<0.05$), in comparison to animals receiving only testosterone (controls) ($p<0.05$).

Influence of the *Serenoa repens* extract on *RafA, MAPK1,* and *MAPK3* transcription

Differences of the mRNA levels in each of the analyzed groups are presented in Table II and Figure 1.

In rats receiving *Serenoa repens* extract, the level of *RafA*, *Mapk1* and *Mapk3* mRNAs was elevated by over 153%, 573.6%, and 631%, respectively ($p<0.05$). The largest elevation of the studied transcripts – by ca. 403% for a *RafA* mRNA ($p=0.124$) and by 1404.2% and 1851.4% in the case of *Mapk1* and *Mapk3* mRNAs, respectively ($p<0.05$), was observed in testosterone-induced rats in comparison to controls. In animals receiving concomitantly testosterone and *S. repens* extract, the level of the studied *Mapk1* and *Mapk3* mRNAs increased by 43.8% and 29.6%, respectively ($p<0.05$), while the *RafA* transcription slightly decreased (by 8.9%) ($p<0.05$), in comparison to animals receiving testosterone only (controls) ($p<0.05$).

Discussion

Understanding the sex-steroid-dependent cellular nongenomic signaling pathway is crucial for better explanation of the molecular background of hormone-dependent diseases and steroid mimetics during induction and progression of carcinogenesis, especially in estrogen-dependent tissues [3, 6, 18]. Molecular studies suggest that changes in mitogen-activated protein kinases (MAPK) phosphorylation/dephosphorylation cascade may be a part of the mechanism which can lead to chemoresistance during cancer pharmacotherapy, i.e. breast cancer, for example lowering the efficacy of tamoxifen treatment [8, 18, 19, 20]. The mechanism, proposed as the one responsible for selective estrogen receptor modulators resistance, is based on tissue-specific cross-talk of MAP kinases and estrogen receptors inducing their phosphorylation and activation, additional adaptive increase in aromatase activity in tumor cells, which subsequently may cause cells to be more responsive to growth effects of estrogen and, consequently, more responsive to the partial agonist activity of tamoxifen [20, 21].

The use of botanical and dietary supplements among menopausal women has increased considerably in recent years [14, 22-24]. In our study, we demonstrated that extracts of *Serenoa repens* and *Epilobium angustifolium* may influence

Table I. The effect of *Epilobium angustifolium* extract on the transcription of *RafA, Mapk1, Mapk3* mRNA.

Group	*RafA* mRNA (%±SEM)	P	*Mapk1* mRNA (%±SEM)	P	*Mapk3* mRNA (%±SEM)	P
E	253.1±0.13	0.076	640.6±0.23	0.009	564.5±0.08	0.017
T	502.9±1.53	0.124	1504.2±3.65	0.007	1951.4±6.9	0.018
TE	500±1.47	<0.001	3312.5±4.23	<0.001	1761±2.84	<0.001
Ratios of changes in mRNA level						
T/K	↑ 4.3		↑ 14.04		↑ 17.51	
E/K	↑ 1.14		↑ 5.4		↑ 4.64	
TE/T	↓ 0.13		↑ 0.52		↑ 0.2	

Transcription changes in all analyzed groups (n=10) were compared to control animals (expression was set on 100%). Results were presented as mean [%] ± SEM of each group; K - control group. E – animals treated with *Epilobium angustifolium* extract; T – testosterone treated animals; TE – testosterone + *E. angustifolium* extract treated rats; p≤0.05 values were considered as statistically significant.
"↑" - elevation of mRNA level in comparison to the control group; "↓" - decrease of mRNA level in comparison to the control group.

Table II. The effect of *Serenoa repens* extract on transcription of *RafA, Mapk1, Mapk3* mRNA.

Group	*RafA* mRNA (%±SEM)	P	*Mapk1* mRNA (%±SEM)	P	*Mapk3* mRNA (%±SEM)	P
S	253.1±0.46	0.016	673.61±1.1	0.001	731±0.77	0.016
T	502.9±1.53	0.124	1504.2±3.65	0.007	1951.4±6.9	0.018
TS	458.3±0.77	0.001	2162.5±2.92	<0.001	2528.9±1.76	<0.001
Ratios of changes in mRNA level						
T/K	↑ 4.3		↑ 14.04		↑ 17.51	
S/K	↑ 1.53		↑ 5.74		↑ 6.31	
TS/T	↓ 0.09		↑ 0.44		↑ 0.3	

Transcription changes in all analyzed groups (n=10) were compared to control animals (expression was set on 100%). Results were presented as mean [%] ± SEM of each group; K - control group; S – animals treated with *Serenoa repens* extract; T – testosterone treated animals; TS – testosterone + *S. repens* extract treated rats; p≤0.05 values were considered as statistically significant.
"↑" - elevation of mRNA level in comparison to the control group. "↓" - decrease of mRNA level in comparison to the control group.

non-genomic signaling pathway via expression changes of genes encoding signaling kinases, including *RafA* and *MAP* proteins. In our opinion, this study confirmed the androgen-dependent nature of transcriptional machinery regulating the mRNA synthesis of the studied genes. Such confirmation is especially visible in our experimental model in all testosterone-induced rats, in which mRNA levels were higher than in control animals. An elevation of the studied mRNA levels is also noticeable in the two groups receiving *Serenoa repens* and *Epilobium angustifolium* extracts, with or without testosterone (Figure 1).

In our opinion, differences between induction of the transcription of the studied mRNAs in the animals administrated both extracts may be mainly due to the competition between major bio-active metabolites contained in those extracts for binding in the structures of enzymes regulating the transcription of the studied mRNAs or in the structures of protein kinases themselves, encoding by the investigated genes. Mainly, phytosterols and/or fatty acids in *Serenoa repens* extract and especially flavonoids (mainly quercetin, myricetin, isoquercetin and their derivatives) and tannins (ellagitannins such as oenothein A and B) in *Epilobium angustifolium* extract may be involved in this competition [15, 25, 26].

Analysis of the literature does not deem any results as far as studies determining the changes in mRNA levels of *RAfA, Mapk1* and *Mapk3* kinases in hormone-dependent organs under the influence of both plant extract are concerned. Moreover, there is limited data concerning the effect of the above mentioned major secondary metabolites contained in the investigated extracts on the molecular activity of the studied kinases.

Conclusions

This study refers to a vital aspect of safety of plant extract use in hormonal therapy of sex steroid hormone-dependent organs. Understanding the importance of sex steroid non-genomic signaling cascade in the progression of proliferative changes will allow to include or eliminate the potential compounds acting as inducers or inhibitors of changes in hormone-dependent organs. Furthermore, improved knowledge about the molecular basis of interactions caused by these plant extracts, in the longer term, may significantly contribute to the establishment of their effective and safe doses in prevention and symptomatic treatment of menopausal proliferative lesions.

We also delivered evidence of potential interactions between some herbal extracts with aromatase inhibitors and SERMs via a

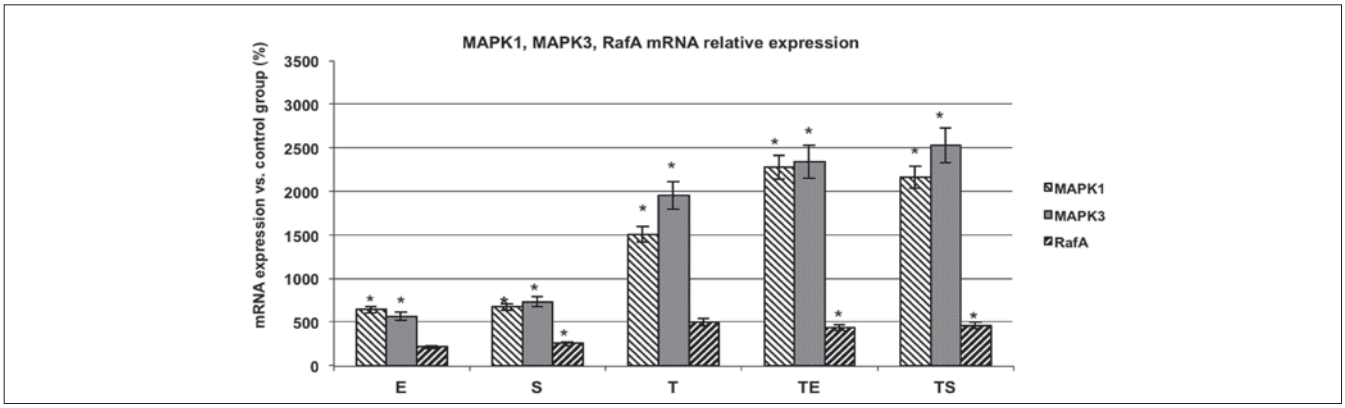

Figure 1. The influence of *Serenoa repens* and *Epilobium angustifolium* extracts on transcription of *RafA, Mapk1, Mapk3* mRNA kinases.
E – animals treated with *Epilobium angustifolium* extract; S – animals treated with *Serenoa repens* extract; T – testosterone treated animals; TE – testosterone + *E. angustifolium* extract treated rats; TS – testosterone + *S. repens* extract treated rats; * p≤0.05 values were considered as statistically significant. All groups (n=10) were normalized vs. the control (expression was set on 100%). Results were presented as mean (%) ± SEM of each group.

non-genomic cellular signaling kinases pathway. The occurrence of such reciprocal influences could disturb the efficiency of breast cancer therapy or other hormone-dependent malignancies.

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