

Effects of Indole-3-Carbinol and Flavonoids Administered Separately and in Combination on Nitric Oxide Production and iNOS Expression in Rats

Evita Rostoka¹, Larisa Baumanė¹, Sergejs Isajevs², Aija Line³, Karina Silina³, Maija Dzintare¹, Darja Svirina², Jelena Sharipova¹, Ivars Kalvinsh¹, Nikolajs Sjakste^{1,2}

¹Latvian Institute of Organic Synthesis, Riga, Latvia

²Faculty of Medicine, University of Latvia, Riga, Latvia

³Latvian Biomedical Research and Study Centre, Riga, Latvia

E-mail: Nikolajs.Sjakste@lu.lv

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Abstract

Beneficial effects of natural compounds are often attributed to modulation of NO production; however effects produced by plant extracts do not correlate with effects of purified components. The goal of our work was to study ability of flavonoids and indole-3-carbinol, as well as their combinations to modify NO production, iNOS gene and protein expression in rat tissues. Baicalein and luteolin decreased NO concentration in both intact and LPS-treated animals. Baicalein decreased iNOS gene expression. Luteolin decreased NO production in the liver and heart and number of iNOS-positive cells in the liver of LPS-treated animals. Combination of the two substances did not decrease the NO synthesis triggered by LPS, although it decreased iNOS gene expression. Quercetin decreased NO production in the heart, kidneys and blood of intact rats, but enhanced the LPS effect in testes, spleen and blood on NO production and iNOS protein expression in the liver. Indole-3-carbinol decreased NO concentration in the cerebellum, blood, lungs and skeletal muscles. The drug enhanced the LPS-triggered increase of NO production in all rat organs. It increased iNOS protein expression in intact liver; however it decreased the LPS-triggered outburst of the enzyme biosynthesis. Combination of indole-3-carbinol with quercetin decreased NO production in LPS-treated animals however it slightly increased iNOS gene expression. Taken together our results suggest that modifications of NO level in tissues by a natural compound cannot be predicted from data about its effects on NOS expression or activity. Combination of substances can produce an effect differing from that of individual substances. This stresses importance of direct measurements of NO in the tissues.

Keywords: Nitric Oxide, Baicalein, Luteolin, Indole-3-Carbinol, Quercetin, Inducible Nitric Oxide Synthase

1. Introduction

Natural biologically active compounds of plant origin including flavonoids are main active substances of traditional Chinese medicines: herbal extracts and similar preparations. Nowadays some of these substances are used in purified form as drugs. Anti-inflammatory activity, antioxidant activities, anticancer activity of phyto-genic antineoplastic agents, and neuroprotective effects of Chinese herbal drugs are in focus of interest of many researchers worldwide [1]. Chinese traditional medicines are known to influence also nitric oxide enzymatic production and NO synthase activity [2]. It is supposed that

flavonoid intake influences mortality from nitric oxide-dependent processes: ischemic heart disease, stroke, diabetes mellitus, and cancer [3], NO production is also modified by chemicals of plant origin [4]. This implies significance of flavonoid and other natural compound uptake for functions of cardiovascular, immune and nervous systems. However biological activity of polyphenol-rich food product does not correlate with effects that could be deduced from effects of individual compounds on NO synthase activity. For example, red wine is known as vasodilator [5], however purified quercetin, that is abundant in red wine inhibits iNOS gene expression [6,7]. It also destabilizes eNOS mRNA [8] and is

even considered to be inhibitor of the NOS enzymatic activity [9]. Nevertheless the compound produces vaso-relaxing effects [10] despite the fact that nitric oxide release in rat aorta is not detected after quercetin administration [11]. In this work we have studied the ability of several natural compounds of plant origin administered separately and in combinations to modify NO production in rat tissues monitored by ESR spectroscopy of Fe (DETC)₂-NO complexes conducted in parallel to evaluation of iNOS gene expression assayed by real-time RT-PCR technique. Combination of direct NO detection in tissues with other approaches characterizing NO production at different levels enabled us to reveal unforeseen effects of presumable NO-donors, anaesthetics and an anti-ischemic drug [12-14]. The same approach was applied this time to natural compounds. Flavonoids luteolin, baicalein and quercetin as well as simple phenolic compound indole-3-carbinol were chosen among many other compounds after a piloting study.

Luteolin, 3', 4', 5, 7-tetrahydroxyflavone is abundant in vegetables: roots of celery, rutabaga, red pepper, spinach and flowering plants: *Ajuga decumbens*, *Taraxacum officinale* (dandelion), *Medicago sativa* (alfalfa). Luteolin is known as dietary compound with antioxidant activity [15]. Baicalein (5, 6, 7-trihydroxyflavone) is found in *Scutellaria baicalensis Georgi* roots. Quercetin (pentahydroxyflavonol) is found in numerous higher plants. This flavonol is abundant in onions, apples, leaf vegetables, beans, tea, red wine, clover, pollen. Indole-3-carbinol (3-indolmetanol) is found in Mustard family plants: (*Brassica sp.*): cabbage, broccoli, Brussels sprouts. The compound is widely studied as chemotherapeutic agent for cancer treatment [16]. Chemical structures of the compounds are given in **Figure 1**. Literature data indicated possible impact of all the three substances on NOS expression and/or NO production [15-22]. The chosen compounds are active substances in several drugs used in Chinese medicine. Quercetin is in important component of Shaofu Zhuyu decoction active extract [23], *Saururus chinensis*, a herb used traditionally in Chinese medicine for treatment of urological diseases [24], together with luteolin it is found in tree peony yellow flowers also widely used in Chinese medicine [25]. Baicalein is an active component of numerous Chinese medicines including Niu Huang Jie Du Pill [26].

The aims of the present work were: 1) To study effects NO production in several organs of intact rats and in LPS model of sepsis; 2) To reveal modifications of NO production by luteolin, baicalein, quercetin and indole-3-carbinol given separately and in combinations in both intact and LPS-treated animals; 3) To study contribution of changes in iNOS gene and protein expression in modifications of NO production by natural compounds and their modifications.

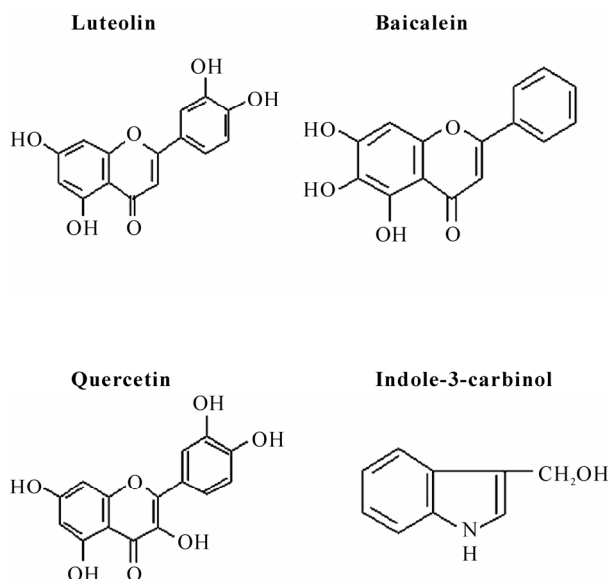


Figure 1. Chemical structures of luteolin, baicalein, quercetin and indole-3-carbinole.

2. Material and Methods

2.1. Chemicals

Indole-3-carbinol, quercetin, baicalein and luteolin were purchased from Dayang Chemical Co., LTD (Hangzhou, China). Lipopolysaccharide, diethylthiocarbamate, ferrous sulfate, sodium citrate, TRI reagent and all other chemicals were from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

2.2. Experiment Design and Drug Administration

Animals were obtained from the Laboratory of Experimental Animals, Riga Stradins University, Riga, Latvia. All experimental procedures were carried out in accordance with guidelines of the Directive 86/609/EEC "European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes" (1986) and were approved by the Animal Ethics Committee of the Food and Veterinary Service (Riga, Latvia).

Wistar male rats, each weighing 215.00 ± 5.63 g at the beginning of the experiments, were used in all the work. The environment was maintained at a temperature of $22 \pm 0.5^\circ\text{C}$ with a 12-h light/dark cycle. The animals were fed a standard laboratory diet. Description of the experimental groups is given in **Table 1**. In NO production experiments substances were administered *per os* in concentrations indicated in the **Table 1**. 3.5 hours after substance administration spin trap was injected, after 30 min

Table 1. Description of groups and experiment design.

Group number	Number of animals	Substance/dose	LPS	Parameter studied	Organs studied
1.	24	-	-	NO production	Brain cortex, liver, heart, kidney, blood, lungs
2.	9	Indole-3-carbinol (50 mg/kg)	-	NO production	Brain cortex, liver, heart, kidney, blood, lungs
3.	5	Luteolin (50 mg/kg)	-	NO production	Brain cortex, liver, heart, kidney, blood, lungs
4.	6	Quercetin (50 mg/kg)	-	NO production	Brain cortex, liver, heart, kidney, blood, lungs
5.	5	Baicalein (50 mg/kg)	-	NO production	Brain cortex, liver, heart, kidney, blood, lungs
6.	28	-	10 mg/kg	NO production	Brain cortex, cerebellum, liver, heart, kidney, blood, lungs, testes, spleen, skeletal muscles
7.	12	Indole-3-carbinol (50 mg/kg)	10 mg/kg	NO production	Brain cortex, cerebellum, liver, heart, kidney, blood, lungs, testes, spleen, skeletal muscles
8.	6	Luteolin (30 mg/kg)	10 mg/kg	NO production	Brain cortex, cerebellum, liver, heart, kidney, blood, lungs, testes, spleen, skeletal muscles
9.	6	Quercetin (50 mg/kg)	10 mg/kg	NO production	Brain cortex, cerebellum, liver, heart, kidney, blood, lungs, testes, spleen, skeletal muscles
10.	6	Baicalein (30 mg/kg)	10 mg/kg	NO production	Brain cortex, cerebellum, liver, heart, kidney, blood, lungs, testes, spleen, skeletal muscles
11.	8	Baicalein (30 mg/kg) Luteolin (30 mg/kg)	10 mg/kg	NO production	Brain cortex, cerebellum, liver, heart, kidney, blood, lungs, testes, spleen, skeletal muscles
12.	6	Quercetin (50 mg/kg) Indole-3-carbinol (50 mg/kg)	10 mg/kg	NO production	Brain cortex, cerebellum, liver, heart, kidney, blood, lungs, testes, spleen, skeletal muscles
13.	20	-	-	iNOS mRNA and protein expression	Liver, brain cortex
14.	3	Indole-3-carbinol (50 mg/kg)	-	iNOS mRNA and protein expression	Liver, brain cortex
15.	3	Luteolin (50 mg/kg)	-	iNOS mRNA and protein expression	Liver, brain cortex
16.	3	Quercetin (50 mg/kg)	-	iNOS mRNA and protein expression	Liver, brain cortex
17.	3	Baicalein (50 mg/kg)	-	iNOS mRNA and protein expression	Liver, brain cortex
18.	3	Quercetin (50 mg/kg) Indole-3-carbinol (50 mg/kg)	-	iNOS mRNA and protein expression	Liver, brain cortex
19.	3	Baicalein (50 mg/kg) Luteolin (50 mg/kg)	-	iNOS mRNA and protein expression	Liver, brain cortex
20.	21	-	10 mg/kg	iNOS mRNA and protein expression	Liver, brain cortex
21.	6	Indole-3-carbinol (50 mg/kg)	10 mg/kg	iNOS mRNA and protein expression	Liver, brain cortex
22.	6	Luteolin (50 mg/kg)	10 mg/kg	iNOS mRNA and protein expression	Liver, brain cortex
23.	6	Quercetin (50 mg/kg)	10 mg/kg	iNOS mRNA and protein expression	Liver, brain cortex
24.	6	Baicalein (50 mg/kg)	10 mg/kg	iNOS mRNA and protein expression	Liver, brain cortex
25.	6	Quercetin (50 mg/kg) Indole-3-carbinol (50 mg/kg)	10 mg/kg	iNOS mRNA and protein expression	Liver, brain cortex
26.	6	Baicalein (50 mg/kg) Luteolin (50 mg/kg)	10 mg/kg	iNOS mRNA and protein expression	Liver, brain cortex

rats were decapitated under slight ether narcosis. In several groups lipopolysaccharide (10 mg/kg) was intraperitoneally injected to rats, substances or their combinations were administered *per os* in the same time, spin traps were administered 3.5 hours later, 30 minutes after spin trap injection rats were decapitated under slight ether narcosis. In additional piloting series of experiments (not shown) iNOS inhibitor AMT (2 mg/kg), was administered intraperitoneally shortly before spin-trap administration, 30 minutes later rats were decapitated. For real-time PCR and immunochemistry rats were decapitated under slight ether narcosis; liver tissue was taken for RNA extraction and immunohistochemical examination. Brain cortex tissue was also taken from some animals for immunohistochemistry. Natural compounds and LPS were administered following the above time schedule.

2.3. Administration of Spin Trap Agents

To determine production level of nitric oxide in the tissues we used ESR spectroscopy of paramagnetic Fe-diethylthiocarbamate–nitric oxide complex (Fe (DETC)₂-NO) [27]. Spin traps were administered 30 minutes before the sacrifice. Rats were administered 400 mg/kg of the nitric oxide scavenger diethylthiocarbamate via intraperitoneal injection and ferrous citrate subcutaneously (40 mg/kg ferrous sulphate + 200 mg/kg sodium citrate). Diethylthiocarbamate binds ferrous ion, the resulting complex traps nitric oxide converting to the paramagnetic Fe (DETC)₂-NO complex that is detected by ESR spectroscopy.

2.4. Sacrifice, Organ Dissection and Sample Preparation for Electron Paramagnetic Resonance Spectroscopy

Following the drug and spin trap administration the rats were decapitated under slight ether anesthesia, samples of brain cortex, cerebellum, myocardium tissue, liver, kidney, testes, skeletal muscles, lungs and blood were compacted in a glass tube 30 mm in length with inner diameter 4 mm and immediately frozen in liquid nitrogen. Before recording the ESR spectra, the specimen was placed in a quartz finger Dewar flask ER 167 FDS-Q (Bruker, Karlsruhe, Germany) filled with liquid nitrogen.

2.5. ESR Measurements

ESR spectra were recorded in liquid nitrogen using an ESR spectrometer “Radiopan” SE/X2544 (Radiopan, Poznan, Poland). The conditions of the electron paramagnetic resonance measurements were: operation at X-band, 25 mW microwave power, 100 kHz modulation frequency, 5 G modulation amplitude, receiver gain 0.5×10^4 , and

time constant 1 s. Spectra were recorded for 4 minutes. The nitric oxide content in the samples was evaluated from the height of the third component of the NO signal at $g = 2.031$.

The NO concentration (ng/g of tissue) was calculated on the basis of calibration curves as described previously. Briefly, different quantities of NaNO₂ (final concentrations 10, 20, 30, 40, 60, 100 M) were mixed with DETC (33 mg/mL) and FeSO₄·7H₂O (3.3 mmol/L), an excess of Na₂S₂O₄ (2 mol/L) was added to the mixture. The EPR spectra were taken as described above.

Further details are given in our previous publications [12-14,28-30].

2.6. RNA Extraction and cDNA Preparation

Total RNA was isolated from liver and brain cortex using TRI reagent (Sigma Aldrich, Taufkirchen, Germany). DNA contaminations were removed with RNA-free kit (Ambion, Austin, TX, USA). The resulting RNA quantity and purity were determined by spectrophotometry, integrity of RNA molecules was monitored by gel electrophoresis, and only specimens with well-pronounced rRNA bands were taken for reactions. RNA (2 µg) was reverse-transcribed using a random hexamer primer (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas, Vilnius, Lithuania) to obtain cDNA.

2.7. Real Time RT-PCR

The mRNA expression rates of the brain cortex, liver iNOS and reference gene were determined using the SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the instructions provided by the manufacturer. Amplification and detection of specific products were performed on a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following temperature-time profile: one cycle of 95°C for 10.00 min; and 40 cycles of 95°C for 0.15 min, 60°C for 1.00 min. To check specificity of amplification products, the dissociation curve mode was used (one cycle at 95°C for 0.15 min, 60°C for 1 min and 95°C for 0.15 min). To evaluate the suitability of candidates as reference genes, we applied the GeNorm program [31]. Primers were designed using Primer3 software. The primers were supplied by Metabion international AG, Germany. The 2^{-ΔΔCT} method was applied for analysis of the results. Primer sequences for iNOS gene were 5'-GCTACACTTCCAACGCAACA-3' for forward and 5'-CATGGTGAACACGTTCTTGG for reverse primer, the expected size of the product was 116 bp. RNA-polymerase II [32] was used as reference gene (5'-GCCAGAGTCTCCCATGTGTT-3' and 5'-GTCGGTGG GACTCTGTTTGT-3', 135 bp).

2.8. Histological and Immunohistochemical Examination

Paraffin-embedded tissue was cut in 4-micron-thick sections and stained with haematoxylin and eosin for morphological examination. Infiltration of inflammatory cells in brain tissue was assessed in subcortical perivascular, subcortical parenchymal, and intracortical peri-vascular regions (magnification $\times 400$). Perivascular infiltrates were defined as inflammatory cells, which are located not further than three cell layers from blood vessels. Inflammatory cells further than three layers from a blood vessel wall were defined as parenchymal infiltrates. Infiltration of inflammatory cells was assessed according to four score scale: 0—no infiltration; 1—light infiltration; 2—medium infiltration; 3—marked infiltration; 4—very marked infiltration (more than 25% of the total field of vision).

The morphology of liver tissue was evaluated by evaluating the histological activity index (HAI), as described [33]: infiltration of inflammatory cells (0–4 scores); necrosis of hepatocytes around a central vein (0–6 scores); necrosis of hepatocytes and apoptosis in periphery lobules (0–4 scores); inflammatory changes of portal tracts (0–4 scores).

Tissue sections were stained for visualization of iNOS positive cells by an immunohistochemical approach as previously described [34]. Briefly, antigen retrieval was achieved by treatment in a microwave oven for 20 min at 300 W in citrate buffer, pH = 6.0. Endogenous peroxidase activity was blocked by 0.5 % H_2O_2 for 10 min. Nonspecific primary antibody binding was blocked by serum-free protein block for 10 min. Rabbit polyclonal active iNOS antibody Abcam Inc. (Cambridge, MA, USA) was applied in 1:200 dilution and incubated for 1h at room temperature in a humidified chamber. Detection of primary antibody binding was performed using specific peroxidase conjugated polyclonal goat anti-rabbit IgG (1:100 for 30 min) and subsequently peroxidase conjugated polyclonal rabbit anti-goat IgG (1:100 for 30 min). The immunoperoxidase color reaction was developed by incubation with diaminobenzidine (7 min). A negative control without primary antibody was included in each staining run. iNOS positive cells were counted in twenty high-powered fields at magnification $\times 400$. All cell counts were expressed as cells per square millimeter. For morphological examination, at least 3 replicate measurements of iNOS positive cells were performed by the same observer in 10 randomly selected slides, and the intraobserver reproducibility was assessed with the coefficient of variation and with the interclass correlation coefficient. The intraobserver coefficient of variation was 4%, and the intraobserver correlation coefficient was 0.94.

2.9. Statistical Analysis

Results were expressed as mean \pm SD. The significance of differences in NO concentration and iNOS expression between groups was evaluated according to Student's unpaired *t*-test, the Mann-Whitney U test was used for quantification of immunohistochemical experiments. Results were considered to be significant when *P* was less than 0.05.

3. Results

3.1. Effects of Natural Compounds and their Combinations on NO Production in Intact and LPS-Treated Rats

In order to test the ability of the natural compounds to modify NO production in animals the radical concentration was monitored in several rat organs and tissues. Data are summarized in **Figure 2**. ESR spectra of the different organs had a typical Cu-DETC spectrum shape with a superposed $\text{Fe}(\text{DETC})_2$ -NO peak, spectra were published previously [14]. The NO production reached the highest levels in the brain cortex, liver, lungs, and blood **Figure 2**. The NO production in heart and kidneys was an order of magnitude lower.

When control group of animals was compared to animals treated with natural substances it turned out that baicalein decreased NO concentration in heart, kidney, liver and lungs (**Figure 2**). Luteolin decreased NO production in the liver and heart. Quercetin induced significant decrease of NO production the heart, kidneys and blood. Indole -3-carbinol caused a significant decrease of NO production in the cerebellum, spleen, blood, lungs and skeletal muscles.

In the following set of experiments the eventual activity of the compounds as modifiers of NO production was tested against the background of the iNOS induction. Intraperitoneal injection of LPS to the animals caused a drastic increase of NO production levels in all tissues studied (**Figure 2**). The highest production of nitric oxide was detected in liver, whereas very strong increases in nitric oxide accumulation (50–100 fold compared to control) were observed in heart, blood and kidney. However, the effects of LPS were less pronounced in brain tissues where nitric oxide increased 4–6 times only. Nitric oxide production increase in testes was of comparable magnitude.

Baicalein (30 mg/kg) decreased NO concentration in brain cortex, liver, heart and kidneys. Luteolin (30 mg/kg) decreased NO outburst in all organs except skeletal muscles. In contrast, administration of the indole-3-carbinol (50 mg/kg) enhanced the LPS-induced increase of NO production in all organs except spleen and testes, **Figure 2**. Quercetin (50 mg/kg) produced similar effect: NO

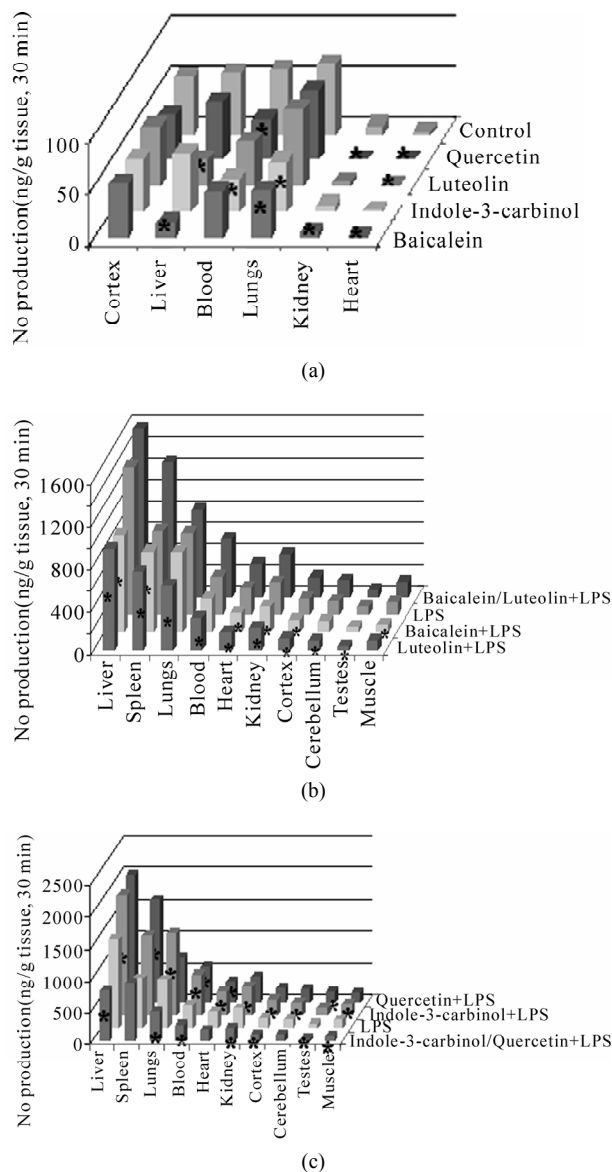


Figure 2. Effects of indole-3-carbinol, luteolin, baicalein quercetin and combinations of indole-3-carbinole + quercetin, baicalein + luteolin on NO production in rat organs of intact (a) and LPS-treated rats (b, c). Stars indicate statistically significant differences ($P < 0.05$) with control (a) or LPS (b, c) groups.

production increased in testes blood and spleen (Figure 2).

The observed ability of individual compounds to modify the LPS-triggered NO production raised the question about maintenance of the effects in presence of other substances. This approach modeled to some extent administration of flavonoid-containing herbal extracts. Baicalein as NO down-regulating and indole-3-carbinol as NO up-regulating substances were supplemented by luteolin and quercetin that produced NO-decreasing and NO-increasing effects correspondingly. Synergism of the effects was anticipated. Surprisingly, the results indi-

cated adverse effects in both cases. Combination of indole-3-carbinol with quercetin (50/50) decreased NO concentration up-regulated by LPS in many tissues. Similarly, combination of baicalein and luteolin lost the NO-decreasing activity characteristic of individual substances (Figure 2).

To test possible involvement of iNOS in the observed modifications natural substances were combined with iNOS inhibitor AMT (not shown). In intact animals the inhibitor markedly decreased NO production in all organs except the heart, AMT inhibiting action was attenuated by indole-3-carbinol in skeletal muscles. Quercetin also attenuated AMT effects in brain cortex, testes, blood and muscles. Luteolin slightly interfered with AMT action. These results indicated possible involvement of iNOS in effects of the compounds; this encouraged us to test ability of the substances to modify iNOS gene expression in rat liver.

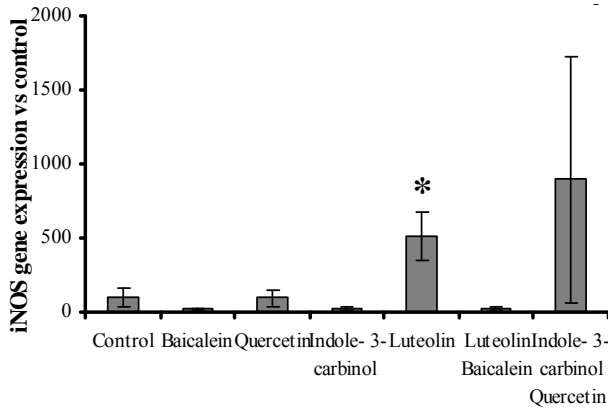
3.2. Effects of Natural Compounds on iNOS Gene mRNA Expression in Intact and LPS-Treated Rats

No influence of indole-3-carbinol on iNOS expression in liver was observed (Figure 3). Surprisingly, luteolin up-regulated the gene expression. Baicalein decreased level of the gene expression. Level of transcription was still decreased when baicalein was given in combination with luteolin. Quercetin did not produce any significant effect on iNOS gene transcription, a tendency for increase was observed when it was given in combination with indole-3-carbinol (Figure 3(a)).

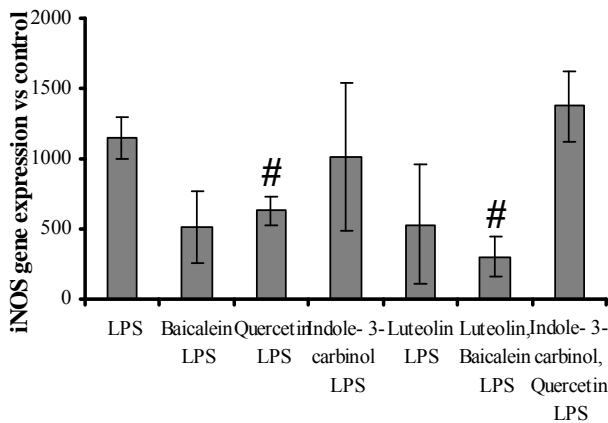
The LPS effect on the gene expression in the liver was drastic (Figure 3(b)) as it could be predicted from the increase of NO production (3442.82 ± 761.24). However the effect was not well-reproducible between individual animals. Quercetin (50 mg/kg) significantly decreased the LPS-triggered iNOS mRNA expression. Enhancement of the iNOS mRNA expression by indole-3-carbinol was observed ($7715.01 \pm 1877.35\%$ compared to control, the result is not statistically significant). The same trend was observed when indole-3-carbinol was supplemented by quercetin (50 mg/kg). Baicalein somewhat decreased the gene expression triggered by LPS, the effect was better pronounced if it was combined with luteolin (Figure 3(b)).

3.3. Effects of Natural Compounds on iNOS Protein Expression in Intact and LPS-Treated Rats

Data on effects of the tested compounds on number of iNOS positive cells in rat liver and brain cortex are given in Figures 4 and 5. Interestingly, indole-3-carbinol pro-



(a)

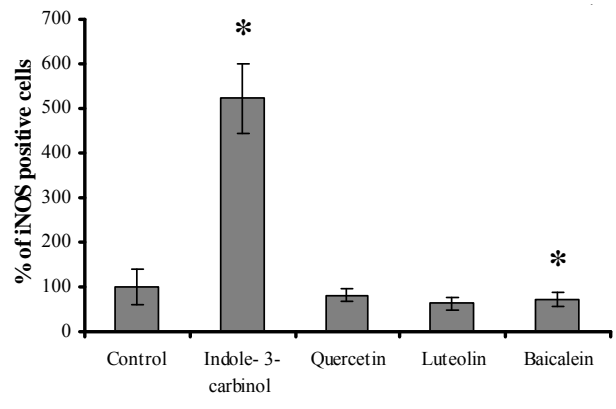


(b)

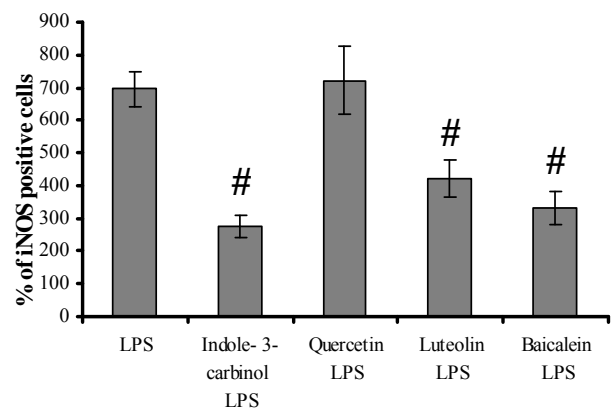
Figure 2. Effects of indole-3-carbinol, luteolin, baicalein, quercetin and combinations of indole-3-carbinol + quercetin, baicalein + luteolin on iNOS gene expression in rat liver. Results are presented as percentage vs average of the control. (a) intact animals; (b) LPS-treated animals. All compounds were administered in dose 50 mg/kg. * $-P < 0.05$ versus control group, # $-P < 0.05$ versus LPS group.

duced statistically significant increase of the protein expression; an effect is coherent with ESR data (Figure 2), the substance increased iNOS expression also in brain cortex (Figure 5(f)). Quercetin (50 mg/kg) and luteolin did not modify the protein expression level. Baicalein manifested a tendency to decrease number of iNOS-positive cells in the liver.

LPS significantly increased the number of iNOS positive cells in liver and brain cortex tissue. Indole-3-carbinol decreased outburst of iNOS protein translation triggered by LPS both in liver tissue (predominantly in Kupfer cells) and brain cortex tissue (Figures 5(c) and (g)), in this case immunohistochemistry data are in contradiction with ESR data indicating enhancement of LPS effects by the compound. Baicalein significantly decreased the LPS effect. On the contrary, quercetin enhanced expression of the enzyme in the liver of LPS-treated animals; this effect followed the trend observed in ESR experiments.



(a)



(b)

Figure 3. Effects of indole-3-carbinol, luteolin, baicalein and quercetin on number of iNOS-positive cells in rat liver. Results are presented as percentage vs average of the control. (a) intact animals; (b) LPS-treated animals. All compounds were administered in dose 50 mg/kg. * $-P < 0.05$ versus control group, # $-P < 0.05$ versus LPS group.

4. Discussion

4.1 Baicalein and luteolin

According published data baicalein suppresses iNOS gene expression in glia [35-37] and macrophages [38, 39] the inhibiting effect is achieved by decrease of lipoxine synthesis. In blood vessels this compound acts as antagonist of nitric oxide, it inhibits the soluble guanylate cyclase [40]. Baicalein depresses also the smooth muscle iNOS [41]. Luteolin is also known as a weak inhibitor of iNOS expression, but it is not capable to inhibit the enzyme activity [17-20]. Some authors find that luteolin stimulates eNOS gene expression [21]. Apparently our results about decrease of NO concentration in some tissues after luteolin and baicalein administration are in good agreement with literature data, as this effect was observed both in intact and LPS-treated animals. Unexpectedly combination of the two compounds did not produce

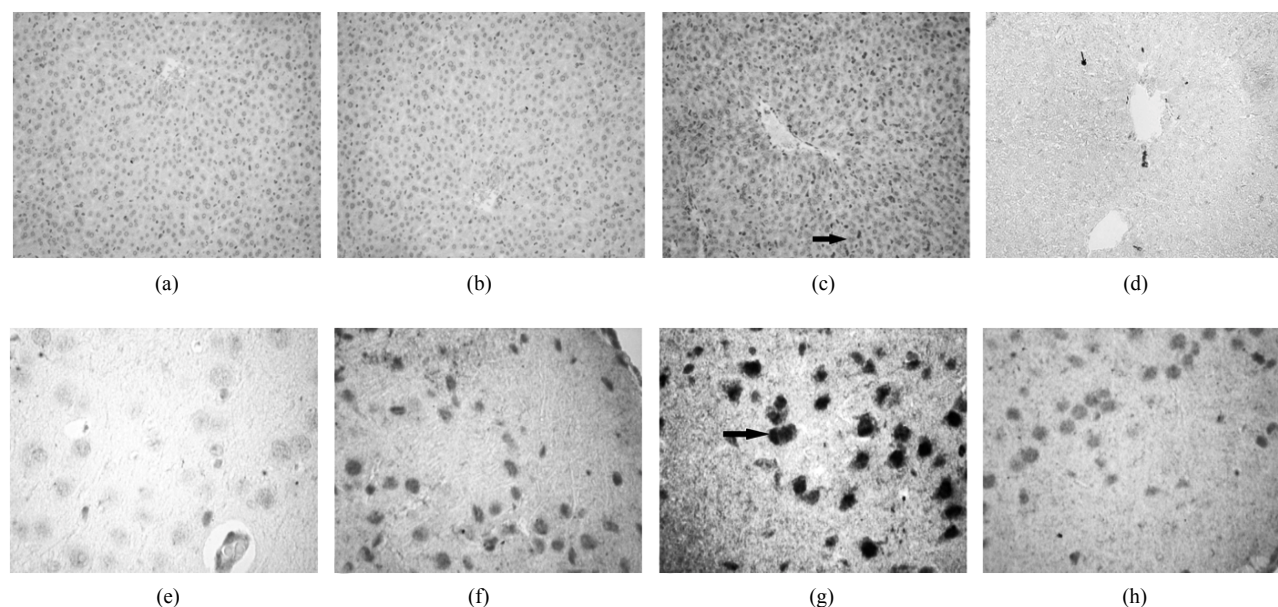


Figure 5. Photomicrographs of in rat liver tissue stained immunohistochemically with iNOS antibody (a-d), and brain cortex tissue (e-h). (a,e)-control, (b,f)-indole-3-carbinol, (c,g)-LPS at dose 10 mg/kg; (d,h)-simultaneous administration of indole-3-carbinol and LPS. Arrows indicate iNOS positively stained cells. Magnification at $\times 100$ (a-d) and at $\times 400$ (e-h).

this effect. iNOS gene and protein expression was decreased by baicalein, this effect persisted when luteolin was administered to the same animal. Interestingly, *Scutellaria baicalensis* extract, *i.e.* combination of baicalein with other compounds increases NO synthesis in induced macrophages [42]. Interesting explanation can be proposed on the basis of hypothesis about nitrite protonation in acidic medium with generation of nitric acid. In this case flavonoids could reduce the acid to NO and stomach could turn into NO generator [43]. Simpler explanations can be also proposed. We have observed that higher dose of baicalein (100 mg/kg, not shown) produced less pronounced NO-lowering effect compared to a lower dose (50 mg/kg). Probably cytotoxic effect of the substances interferes with NO-modulating activity. Both baicalein and luteolin are inducers of apoptosis, the substances intercalate into DNA, inhibit topoisomerases I and II and DNA polymerase, induce TNF [44]. Thus apoptosis process can be triggered in many cells, NO level would increase as protection reaction against apoptosis [45].

4.2. Indole-3-Carbinol and Quercetin

Indole-3-carbinol is a weak iNOS induction inhibitor [22]. The substance interferes with several signaling pathways [46]. Decrease of NO concentration in blood, lungs and skeletal muscles caused by the substance can also indirectly indicate iNOS-inhibiting activity of the compound. Surprisingly, inverse effect was observed when we assessed number of iNOS-positive cells in the

liver of intact rats. The compound enhanced increase of the gene transcription triggered by LPS in the liver, NO production in liver of intact animals and the LPS-induced increase of NO production in all the organs studied, amazingly, number of iNOS-positive cells in the liver of LPS-treated animals dropped down after administration of the substance. Thus the substance can either increase or decrease NO production in rat tissues. The effect depends on the tissue and physiological state of the animal. The NO decreasing activity of indole-3-carbinol can be explained by its capability to reduce NF- κ B DNA binding activity [16,46]. Moreover the substance was shown to inhibit Akt-kinase activity, this leads to decrease of NF- κ B expression [16,46]. Ability of the compound to increase the NO production in some organs, especially in LPS-treated animals could be rather ascribed to antioxidant activity of indole-3-carbinol [22]. Scavenging of reactive oxygen species prevents involvement of NO in interaction with these radicals increasing its bioavailability. This effect is produced by several natural compounds including cocoa polyphenols [47] and resveratrol [48]. Increase of enzyme expression by the drug indicates existence of some mechanism for regulation on transcription level. Enhancement by the substance of nitric oxide production induced by lipopolysaccharide upregulation of protein kinase C is also quite possible [49,50].

Quercetin decreased NO concentration in the heart, kidneys and blood; however it enhanced the LPS effect in testes, blood and spleen. The substance attenuated the LPS effects on the level of iNOS gene expression, however it enhanced these effects on protein expression level. Data about quercetin impact on NO synthesis are rather

contradictory. The substance inhibits iNOS induction, the effect is better detectable in *in vitro* cultured cells [6,7,51]. *In vivo* quercetin did not decrease NFκB activation in kidney cortex [52]. In systems, where iNOS expression decrease by quercetin was observed, the flavonoid did not down-regulate NFκB activation [7,53]. Probably, quercetin interferes with tyrosin kinase-mediated pathways [54], and decreases TNFα expression [55]. However other authors observed the quercetin-induced inhibition of IκBα (*inhibitor of kappa B alpha*) degradation via depression of IκB kinase activity, this leads to inhibition of NFκB [56]. Other reports inform about prevention and/or inhibition of IκB phosphorylation [57, 58], depression of NFκB activation by interleukin [59] and hydrogen peroxide [60] produced by the flavonoid. Inhibition of iNOS expression by quercetin is often ascribed to inhibition of the NFκB pathway [61,62]. Interestingly, some authors [63] find out that quercetin does not inhibit iNOS gene expression; however it inhibits iNOS enzyme expression. We came up to quite an opposite conclusion; however both the cited and our data indicate separate mechanisms for regulation of the iNOS gene and protein expression by this substance. Quercetin also destabilizes eNOS mRNA [8]. There are even data about ability of the substance to inhibit NOS enzymatic activity [9]. In the same time quercetin protects epithelium against lesions produced by NOS inhibitors [64] and stimulates NO synthesis in leukemia cells as protection reaction against induction of apoptosis [45]. Quercetin is considered to be the main active compound of red wine; some authors have observed NO-dependent vasorelaxation induced by quercetin [10]. Supplementation of diet with quercetin favors NO production in endothelium [5]. However the nitric oxide release from endothelium was not detected in special studies [11]. Quercetin is also one of the active substances of the *Gingko biloba* extracts, both purified quercetin and *Gingko biloba* extracts (*i.e.* mixture of quercetin with other natural compounds) decrease LPS-induced iNOS expression, however the extract acts via NF-κB inhibition, but quercetin inhibits TNFα pathway [65,66]. In our studies quercetin increased the LPS-triggered NO outburst in lungs, testes and lungs. Apparently, the NOS-inhibiting effect was not detectable on organism level. Our data contradict formerly published reports about decrease of nitrite production in brain [67] and blood plasma of LPS-treated animals [68] or streptozotocin-treated animals [69] by quercetin. However nitrite and NO production levels do not always correlate. When NO production was assessed by an approach similar to ours the quercetin-induced increase of NO production in rat brain was also observed [70]. Moreover the substance did not modify much iNOS gene expression in both healthy and LPS-treated animals. This effect can be associated with apoptosis-promoting activity of quercetin [71]. Probably simultaneous administration of indole-3-carbinol and quercetin favored

manifestation of NOS-inhibiting activity of quercetin and abolished increase of NO bioavailability produced by indole-3-carbinol, as no decrease of iNOS expression was observed in this case. NO scavenging activity of quercetin [69-73] also could manifest itself in this case.

5. Conclusions

Taken together our results suggest that modifications of NO level in tissues by the studied natural compounds cannot be predicted from data about its effects on NOS expression or activity. Effects of individual compounds are not additive when these are administered in combination. This stresses importance of direct measurements of NO in the tissues using ESR method.

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