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FLAVONOIDS AND NITRIC OXIDE SYNTHASE

Induction of NOS-2 in macrophages and smooth muscles within vascular wall with concomittant suppression of endothelial NOS-3 activity is considered to be a hallmark of vascular inflammation that triggers atherogenesis. Accordingly, drugs designed to reverse these changes should not only support vaning function of NOS-3 but also suppress proinflammatory NO production by NOS-2. It means that using selective inhibitors of induction of NOS-2 (they spare *ex definitione* constitutive activity of NOS-3) is a more rational approach than using "selective" inhibitors of activity of previously induced NOS-2. First of all, those drugs are never sufficiently selective. In our work we tried to identify inhibitors of NOS-2 induction within the group of flavonoids, known stimulators of NOS-3 with putative antiatherogenic effects. Representatives of four main groups of flavonoids: **flavonols** (kaempferol, quercetin, rutin), **flavones** (apigenin, primuletin), **flavanols** (catechine) and **flavanones** (hesperetin, hesperidin, naringenin) were tried on NOS-2 induction and activity in the in vitro model of LPS-treated macrophages (cell line J774.2). While none of these compounds inhibited activity of NOS-2, all with unexpectedly scattered potencies inhibited induction of NOS-2 protein in LPS-treated J774.2 cells, as evidenced by Western blotting technique. Subsequently, RT-PCR and Northern blotting methods revealed that so far the most potent compounds, ***kaempferol*** and ***apigenin***, at micromolar concentrations did inhibit NOS-2 induction at the level of NOS-2 gene transcription. We conclude that some of flavonoids are potent inhibitors of NOS-2 induction. At the same time they may increase endothelial NOS-3 activity. Could these flavonoids become natural parents of future drugs, which will be used for reversal of inflammatory component of atherothrombosis?

Key words: *flavonoids, nitric oxide synthase, macrophages,*

INTRODUCTION

Nitric oxide (NO) is produced from L-arginine by at least three isoforms of NO synthase (NOS) (1). Under physiological conditions, the highly controlled release of low amounts of NO by constitutive NOS in vascular endothelium (NOS-3) dilates blood vessels and inhibits adhesion of leukocytes and platelets to endothelial surface (2, 3). Inducible isoform of NOS (NOS-2) is expressed due

to the action of inflammatory stimuli including cytokines, endotoxin or reactive oxygen species and once induced, it generates large amounts of NO, toxic for bacteria and tumor cells (4). It means that induction of NOS-2 in macrophages plays an important role in host defence (5). However, in several acute inflammatory conditions, such as endotoxic shock, excess of NOS-2-derived NO is detrimental and contributes to hypotension and vascular hyporeactivity (6). Importantly, at the same time NO produced by NOS-3 plays the protective role (7, 8, 9). This Yin-Yang aspect of nitric oxide metabolism could be observed not only in acute inflammatory reactions. Induction of NOS-2 in macrophages and smooth muscles within vascular wall with concomittant suppression of endothelial NOS-3 activity is considered a hallmark of chronic vascular inflammation that triggers atherogenesis (10, 11). Accordingly, using selective inhibitors of induction of NOS-2 (they spare *ex definitione* constitutive activity of NOS-3) is an approach more rational than using "selective" inhibitors of activity of previously induced NOS-2. Unfortunately, adrenocorticoids, currently available powerful inhibitors of NOS-2 induction display a large scale of unwanted effects.

In our work we tried to identify safe inhibitors of NOS-2 induction within the group of flavonoids, plant-derived polyphenolic compounds, known NOS-3 stimulators (12, 13) with recognized antiinflammatory, antiatherogenic and antioxidant activities (14, 15, 16, 17).

Mouse macrophages (cell line J774.2), stimulated with lipopolysaccharide (LPS) were used as a model of NOS-2 induction. Additionally, we tested the influence of flavonoids on NOS-3 activity, by estimating vascular responses to selected flavonoids administered into coronary circulation of isolated guinea pig heart.

MATERIALS AND METHODS

Mouse Macrophage Cell Line J774.2

The mouse macrophage cell line J774.2 was cultured in T75 flasks in DMEM that contained 10% foetal bovine serum supplemented with streptomycin (100 μg ml^{-1}), penicillin (100 U ml^{-1}) and fungizone (0,25 μg ml^{-1}). Flasks were kept at 37 ^{o}C in atmosphere of humidified air containing 5% CO_2. Then, the cells were seeded in 96-well plates and cultured in 200 μl of culture medium until reaching confluence (10^5 cells per well). Additionally, in order to obtain sufficient amount of material for mRNA and protein assays, the cells were cultured in 6-well plates (10^6 cells per well) in 2 ml of culture medium. Nitric oxide synthase (NOS-2) in macrophages was induced by lipopolysaccharide from *Escherichia coli* serotype 0127:B8 (LPS, 1 μg ml^{-1}).

Protocol of experiments

All tested flavonoids, dissolved in dimethylsulphoxide (DMSO) (final DMSO concentration was less than 0,03%) were given in fresh culture medium either 15 minutes prior to, or 1, 2, 4, 6, and 10 hours after LPS (1 μg ml^{-1}) stimulation.

Culture medium and cells were harvested 24 hours after LPS stimulation.

Nitrite concentration in Culture Medium

Nitrite acculmulation in J774.2 cell culture supernatant was measured using the Griess method (18). Briefly, 100 µl of 1% sulphanilamide in 5% phosphoric acid, followed by 100 µl of 0,1% N-(1-naphtyl)-ethylene diamine in 5% phosphoric acid were added to 100 µl of culture medium. After 10 minutes of incubation at 23 °C the absorbance at 550 nm was read. Micromolar concentrations of nitrite were calculated from standard curve constructed with sodium nitrite as a reference compound.

RT-PCR

Total cellular RNA was isolated by single-step, guanidium thiocyanate-phenol-chloroform extraction with the TRIzol® Reagent (Gibco-BRL, USA) according to the manufacturer's instructions. RNA (1 µg) from each sample was reverse-transcribed to complementary DNA (cDNA) using reverse transcriptase (MMLV Reverse Transcriptase, Gibco BRL, USA). The RT master mix consisted of 25 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.25 mM of each deoxyribonucleoside triphosphates (dNTPs) 1 U μl^{-1} RNase Inhibitor, 10 U μl^{-1} MMLW reverse transcriptase and 25 ng μl^{-1} of oligod$(T)_{12-18}$. The final RT reaction volume was 20 µl. The reaction was performed in a thermal cycler Perkin Elmer 9600 at 42°C during 2 h, and the enzyme was then denatured at 99°C for 5 min.

The 384 base pair NOS-2 cDNA fragment and 308 base pair β-actin cDNA fragment were amplified from RT-generated cDNA using specific primer pairs. The following primer sequences (5'→3') were used: for NOS-2, the sense TGG CTT GCC CTT GGA AGT TTC TC and the anti-sense TGT CTC TGG GTC CTC TGG TCA AA, and for β-actin, the sense AGC GGG AAA TCG TGC GTG and the anti-sense AGC GGG AAA TCG TGC GTG. The polymerase chain reaction was performed in a 25- µl reaction volume containing 1 µl RT product (cDNA) and 24 µl PCR master mix (Tris-HCl, pH 8.7, KCl, $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 U HotStarTaq DNA Polymerase (Qiagen, Germany)and 1 µM concentration of each primer. After an initial enzyme activation step ("hot start") for 15 min at 95°C, PCR was carried out (1 min at 94°C, 30 s at 59°C and 30 s at 72°C), followed by a 10-min extension at 72°C.

For each primer pair, control experiments were performed to determine the range of cycles in which a given amount of cDNA would be amplified in a linear fashion: NOS-2, 30 cycles and β-actin, 30 cycles. Semiquantitative analyses of photographs of ethidium bromide- stained DNA gels (2% agarose) were performed with Scion image (Scion Corporation, USA). The data were normalized to transcript levels for the constitutively expressed β-actin gene.

Northern hybridization

The PCR-amplified NOS-2 cDNA fragment and the rat glyceraldehyde 3-phosphate dehydrogenase cDNA probe (Sigma, USA) were used for hybridization. The probes were labeled with [α-^{32}P]dCTP using the Megaprime™ DNA labeling kit (Amersham, UK).

10 µg of total RNA from each sample was separated in a 1.2% denaturing agarose gel and transferred onto a nylon membrane (Nylon membranes, positevely charged, Boehringer Mannheim GmbH, Germany) in 2xSSC buffer, overnight, by capillary action. The RNA was immobilized on the membranes by baking under vacuum at 80°C for 2 h. The membranes were prehybridized in QiuckHyb® Hybridization Solution (Stratagene, USA) at 68°C for 20 min. The RNA molecules on the filter were then hybridized in the same solution with [^{32}P]-labeled probe at the concentration of $1.25x10^6$ cpm ml^{-1} at 68°C for 1 h. After hybridization, the blots were washed twice for 15 min at

room temperature with 2xSSC containing 0.1% (w/v) SDS and once for 30 min at 60°C with 0.1xSSC containing 0.1% (w/v) SDS. The RNA molecules on the filter were first hybridized to NOS-2 probe and after film exposure subsequently underwent rehybridization to GADPH probe to normalize the amounts of analyzed RNAs. Blots were exposed to Kodak BioMax MS-1 film, (Sigma, USA). The Northern blot autoradiographs were scanned and analyzed with Scion image (Scion Corporation, USA).

NOS-2 immunoblotting

24 hours after LPS stimulation the cells were lysed in lysis buffer (1% Triton X-100, 0,1% SDS in PBS containing 1 mM PMSF, 100 μM leupeptin, 50 μM pepstatin A). Protein concentrations of lysates were determined using Bradford method. Samples, containing equal amounts of total protein were mixed with gel loading buffer [50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2mg/ml bromophenol blue] in a ratio 1:1 (v/v) and boiled (4 min). Then samples (30 μg of total protein per lane) were separated on 7,5% SDS-polyacrylamide gels (Mini Protean II, Bio-Rad, USA) using Laemmli buffer system and proteins were semi-dry transferred to nitrocelulose membranes (Bio-Rad, USA). Non-specific binding sites were blocked overnight in 4 °C with 5% non-fat dried milk and the membranes were then incubated 2 hrs in room temperature (RT) with rabbit polyclonal antibody to NOS-2 (1: 2000) (Transduction Laboratories, USA). Bands were detected with alkaline phosphatase-conjugated secondary antibody (1 hr in RT, 1: 5000, Sigma, USA) and developed with BCIP and NBT (Sigma, USA). Rainbow markers (Amersham, USA) were used for molecular weight determinations. Protein bands were scanned and analyzed with freeware Scion image (Scion Corporation, USA).

Cell respiration assay

Cell respiration, an indicator of cell viability, was assessed by mitochondrial-dependent reduction of MTT to formazan (18). Cells in 96-well plates were incubated in 37°C with MTT (0,2 mg ml^{-1} for 60 minutes). Then, culture medium was removed by aspiration and cells were solubilized in DMSO (200 μL). The extent of reduction of MTT to formazan within cells was quantitated by measurement of absorbance at 550 nm.

Langendorff preparation of guinea pig heart

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and the experimental procedure used in the present study was approved by the local Animal Research Committee.

The details of the method were described elsewhere (19). Briefly, guinea pigs of both sex and body weight 300 - 400 g were anaesthetized with pentobarbital (30 - 40 mg kg^{-1} body weight). Their hearts were isolated, washed in ice cold saline, and the coronary circulation was perfused through an aorta under a constant perfusion pressure of 60 mm Hg using Langendorff apparatus of Hugo Sachs Electronics (HSE). The hearts were perfused with Krebs-Henseleit buffer of the following composition (mM): NaCl 118, $CaCl_2$ 2.52, $MgSO_4$ 1.64, $NaHCO_3$ 24.88, K_2P0_4 1.18, glucose 5.55, sodium pyruvate 2.0, equilibrated with 95%O_2 + 5% CO_2 at 37°C in the oxygenator with rotating disc (HSE). The hearts were paced with 273 impulses per min through two platinum electrodes placed in the right atrium. Left ventricular pressure (LVP) was measured using the fluid-filled balloon inserted into the left ventricle and connected to a pressure transducer (Isotec HSE). The end diastolic pressure was adjusted to 5-10 mm Hg. The dP/dt_{max} and dP/dt_{min} values were calculated from LVP signal by an analogue differentiation amplifier (DIF module HSE). Coronary flow (CF) was monitored by Ultrasonic flowmeter (HSE). LVP, dP/dt_{max}, dP/dt_{min} and CF were calibrated once

a day before the experiment and then continuously displayed throughout the experiment and, finally, analysed using special-designed software (PSCF. EXE-IGEL, Poland).

Quercetin, kaempferole, and apigenin (each dissolved in 30% DMSO in 10 μM and 30 μM concentrations) were infused into the coronary circulation of isolated guinea pig either in the absence or in the presence of non-selective NOS inhibitor, L-NAME (100 μM). In paralell experiments, infusion of 30% DMSO at the same rate (approximately of 2 % of the coronary flow: 100 - 300 μl/min) did not influence coronary flow in isolated guinea pig heart.

Statistics

All values in the figures and text are expressed as mean ± s.e.of *n* observations. A one way analysis of variance (ANOVA) followed, if appropriate, by a Bonferroni`s test for multiple comparisons was used to compare means between the groups. A *P* value less than 0,05 was considered to be statistically significant.

Chemicals

Apigenin, kaempferole, quercetin, rutin, primuletin, catechin, hesperetin, hesperidin, naringenin, MTT- [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] BCIP [5-bromo-4-chloro-3indolyl phosphate] and NBT [nitro blue tetrazolium] were from Sigma, USA.

RESULTS

Influence of flavonoids on NOS-2 induction in LPS-treated J774.2 cells

Stimulation of J774.2 cells with LPS (1 μg/ml) caused a significant increase of 24-hr nitrite accumulation in culture medium (from 4,5 ± 0,8 to 51 ± 3,2 μM) (control cells were treated with vehicle, but not with LPS).

A representative of flavonoids, kaempferole (0,1 – 100 μM), administered 15 min prior to LPS (1 μg/ml), inhibited 24-hr nitrite accumulation in dose-dependent manner (*Fig. 1A*). Importantly, kaempferole administered 10 hrs after LPS (1 μg/ml) did not influence 24-hr nitrite accumulation in culture medium of J774.2 cells (*Fig. 1A*).

The inhibition by kaempferole (10 μM) of the increase in 24-hr nitrite formation became progressively weaker when kaempferole was added to the cells at 1, 2, 4, 6 or 10 h after LPS (*Fig. 1B*). Although the administration of kaempferole up to 4 hr after LPS still caused a partial inhibition of 24-hr nitrite accumulation, addition at 6 or 10 hr after LPS had no significant effect on nitrite formation by LPS-treated cells (*Fig. 1B*).

Kaempferole, added 15 minutes prior to LPS (1 μg/ml) dose dependently inhibited NOS-2 protein induction in J774.2 cells (*Fig. 2C*), while it decreased the levels of NOS-2 mRNA in LPS-treated J774.2 cells, as evidenced by RT-PCR and northern blot techniques (Fig. 2A and 2B, respectively).

None of the tested flavonoids affected the viability of J774.2 cells (as determined by the MTT assay) either when given alone or in combination with LPS (data not shown).

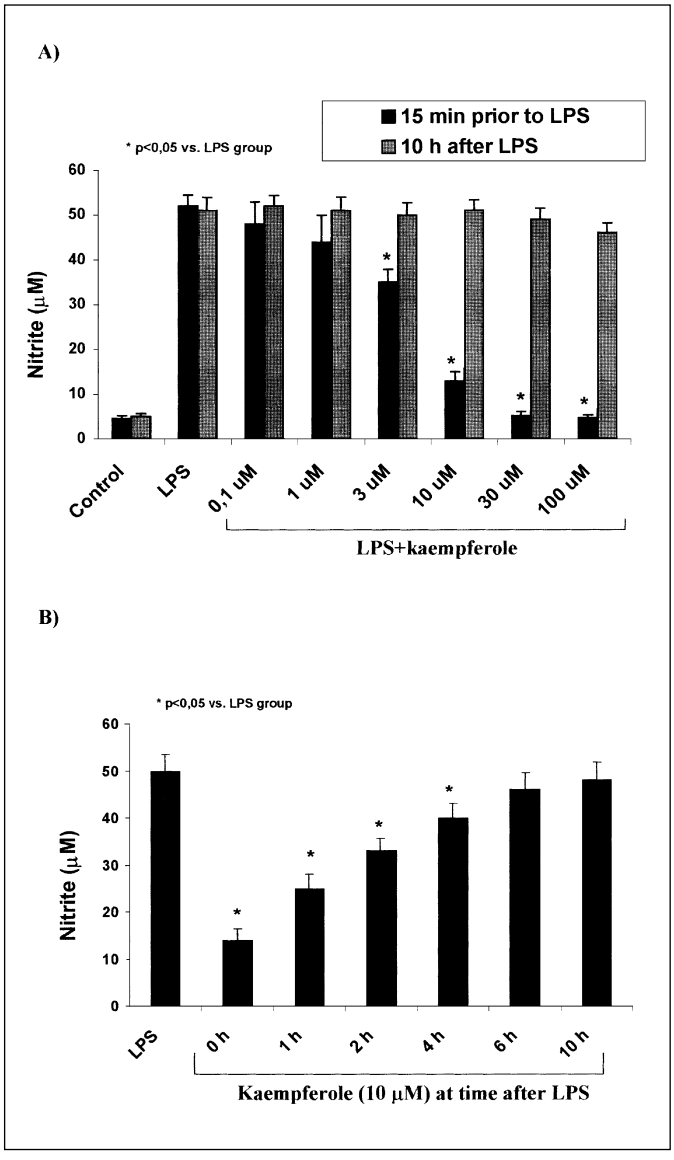


Fig. 1. **(A)** Kaempferole, administered 15 minutes prior to LPS (1 μg ml^{-1}) causes dose-dependent inhibition of nitrite accumulation in culture medium of J774.2 cells. In contrast, kaempferole at any concentration, administered 10 hrs after LPS (1 μg ml^{-1}) did not influence 24-hr nitrite accumulation. **(B)** The inhibition by kaempferole of the nitrite formation was time-dependent. Kaempferole (10 μM) was added to culture medium either together with LPS ((1 μg ml^{-1}) or at 1, 2, 4, 6 and 10 h after LPS-stimulation. Data are expressed as mean ± s.e. of n = 6 independent experiments. *p<0,05 represents significant difference as compared to LPS group.

The pattern of influence on 24-hr nitrite accumulation, NOS-2 mRNA level and NOS-2 protein induction was very similar for all tested flavonoids. However, they showed surprisingly scattered potencies of inhibitory action. Table 1 shows structures, names and concentrations, which inhibit 50 % of 24-hr nitrite accumulation caused by LPS (half-maximal inhibitory concentration, IC_{50}). The most active compounds were kaempferole (IC_{50}=5,7 μM) and apigenin (IC_{50}=6,86 μM) (*Tab. 1*).

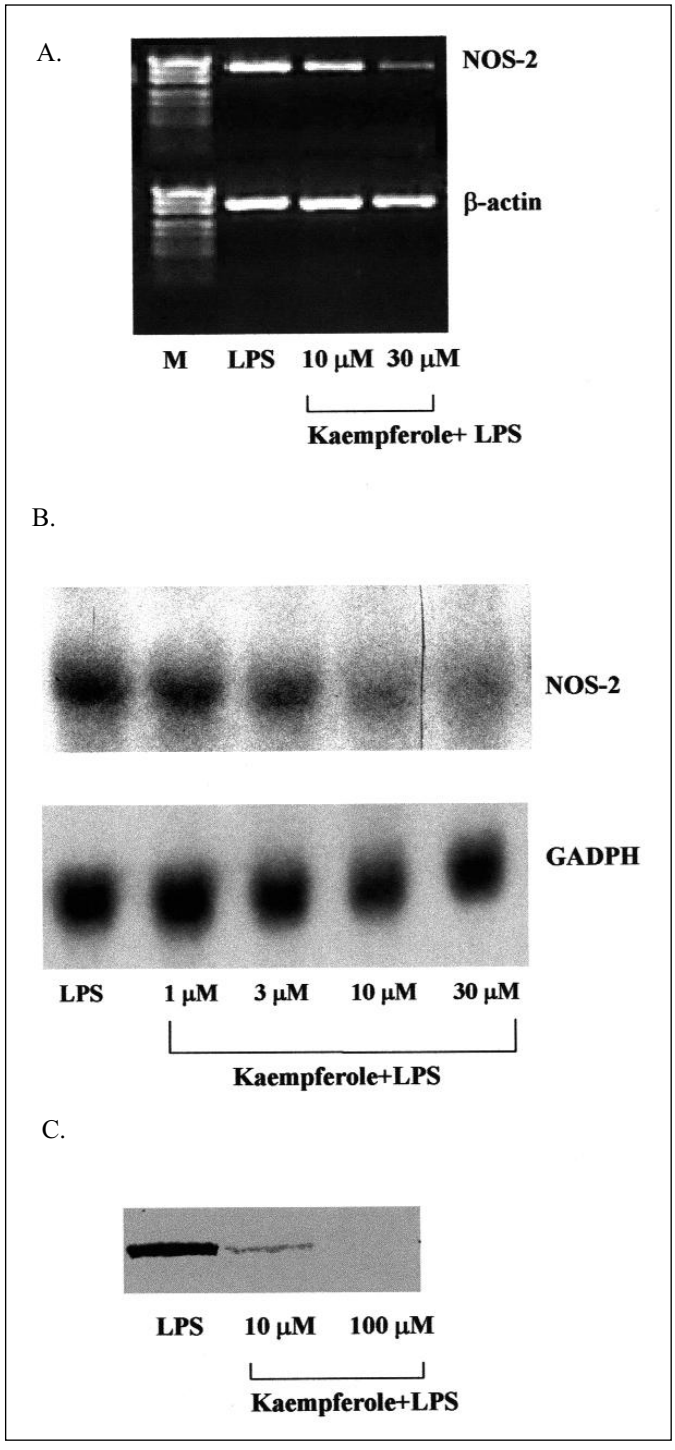
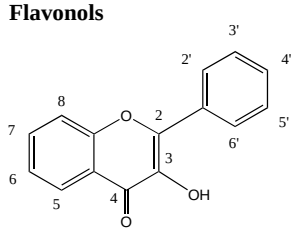
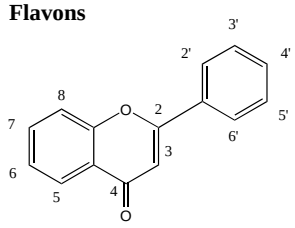
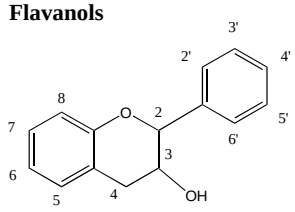
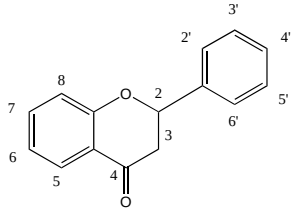


Fig. 2. **(A)** RT-PCR analysis of NOS-2 mRNA and β-actin mRNA isolated from J 744.2 cells. A 384-base pairs (bp) fragment and a 308-bp fragment were amplified for NOS-2 and for β-actin, respectively. Kampferole, dose dependently decreased NOS-2 mRNA level in LPS-stimulated J774.2 cells. **(B)** - northern blot analysis of NOS-2 mRNA expression in J 744.2 cells. Total RNA isolated from cells were hybridized with radiolabeled NOS-2 cDNA probe (upper panel) and reprobed with a radiolabeled cDNA probe for GADPH (lower panel). Kaempferole caused dose-dependent decrease of NOS-2 mRNA levels in LPS-treated (1 μg ml^{-1}) J774.2 cells. **(C)** – immunoblotting of NOS-2 protein. Kaempferole dose dependently inhibitited NOS-2 protein induction in LPS-treated (1 μg ml^{-1}) J774.2.

Table 1. Structures, common names and half-maximal inhibitory concentrations ($IC_{50,}$ μM) of tested flavonoids. IC_{50}- flavonoid concentration, which inhibits 50 % of 24-hr nitrite accumulation in culture medium of LPS-stimulated (1 μg ml^{-1}) J774.2 cells.

Structure	Name	Substituents 3	5	7	3'	4'	5'	IC_{50} (μM)
Flavonols 	**Kaempferole**	OH	OH	OH	H	OH	H	**5,7**
	Quercetin	OH	OH	OH	OH	OH	H	**92,1**
	Rutin	OR	OH	OH	OH	OH	H	**172,0**
Flavons 	**Apigenin**	H	OH	OH	H	OH	H	**6,86**
	Primuletin	H	OH	H	H	H	H	**63,0**
Flavanols 	**Catechin**	OH	OH	OH	OH	OH	H	**191,2**
Flavanons 	**Hesperetin**	H	OH	OH	OH	OCH_3	H	**101,3**
	Hesperidin	H	OH	OR	OH	OCH_3	H	**91,1**
	Naringenin	H	OH	OH	H	OH	H	**59,3**

R-rutinose

Influence of flavonoids on coronary flow in isolated guinea pig heart

In isolated guinea pig heart basal coronary flow and basal contractility (dp/dt) were 9,34 ±1,04 ml/min and 1105 ±56 mmHg/sec, respectively.

Infusion of flavonoids (quercetin, kaempferole, apigenin) into coronary circulation of isolated guinea pig heart for 1 min caused immediate vasodilatation. Quercetin (30μM) appeared to be the most potent vasodilator (*Fig. 3*). Kaempferole (30 μM) dilated coronary vessels of isolated guinea pig heart much stronger than apigenin (30 μM) (*Fig. 3*). Coronary responses to flavonoids were strongly inhibited by L-NAME (100 μM), non-selective inhibitor of NOS activity (*Fig. 3*). Noteworthy, in isolated guinea pig heart L-NAME (100 μM) almost completely abolished coronary response to bradykinin (*Fig. 3*).

DISCUSSION

Here we identified flavonoids as inhibitors of NOS-2 induction in LPS-treated mouse macrophages. The most potent compounds, kaempferol and apigenin, at

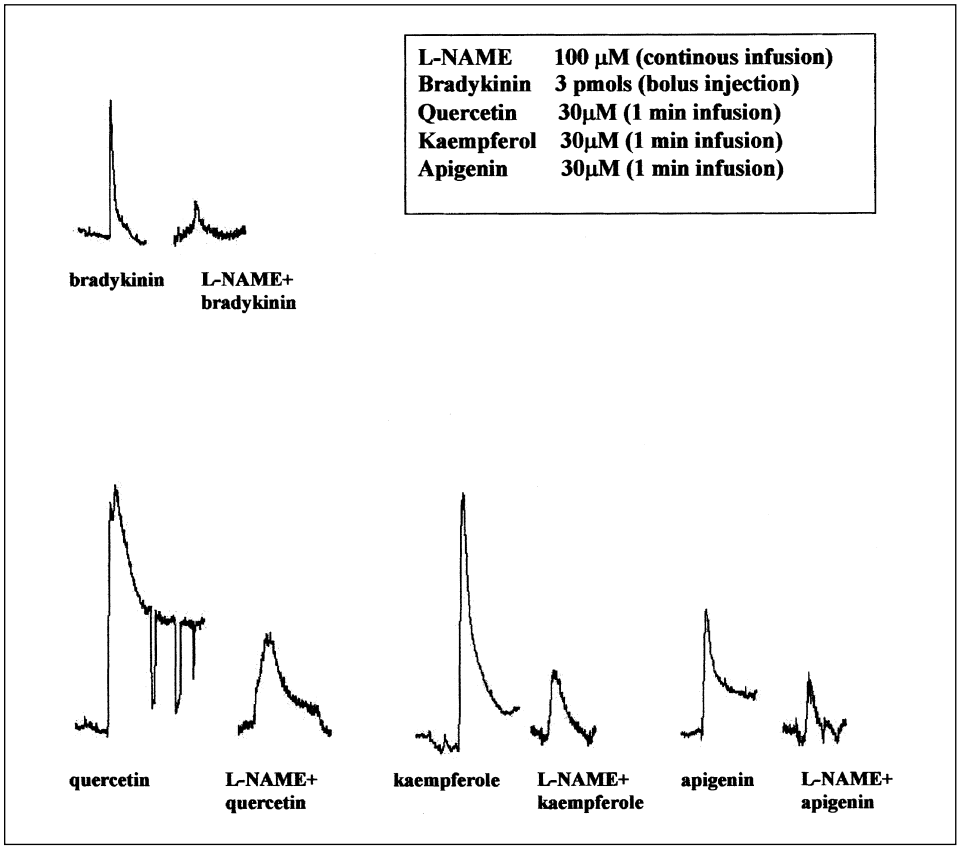


Fig. 3. Samples of original tracings of vasodilatations of isolated guinea pig heart coronary vessels caused by quercetin (30 μM), kaempferole (30 μM) and apigenin (30 μM) and by standard endothelium-dependent vasodilator – bradykinin (3 pmoles, bolus injection). L-NAME (100 μM) strongly inhibited either flavonoid-induced vasodilatations or response to bradykinin.

micromolar concentrations inhibited NOS-2 induction at the level of NOS-2 gene transcription.

The model of LPS-treated J774.2 macrophages is widely used in studies of mechanisms of NOS-2 induction (18, 20, 21). In our hands, activation of J774.2 macrophages with LPS resulted in accumulation of nitrite in culture medium. This accumulation of nitrite reflected NO production due to induction of NOS-2 in LPS-treated cells (18). Flavonoids dose-dependently inhibited accumulation of nitrite in supernatant of LPS-stimulated J774.2 cells. This effect was not related to their cytotoxic action, as none of the flavonoids affected the viability of J774.2 cells (as determined by the MTT assay). It shows that flavonoids decreased nitrite formation due to the inhibition of NOS-2 activity and/or inhibition of induction of the enzyme. However, action of flavonoids was lost when the compounds were given 6 or 10 hours after LPS. As in our model the stimulation of J774.2 cells for 10 hours results in nearly maxiuml expression of NOS-2 (data not shown), this finding shows that flavonoids inhibit the induction, but not the activity of NOS-2.

Indeed, pretreatment with flavonoids - dose dependently, although with unexpectedly scattered potencies - inhibited NOS-2 mRNA and protein expression in LPS-stimulated J774.2 cells. The half-maximal inhibitory concentrations and structures of the tested flavonoids were shown in the *table 1*.

The strongly active flavonoids, kaempferole and apigenin possessed a C 2, 3 double bond. Flavanone derivatives, which have not C 2, 3 double bond, appeared to be very weak inhibitors of NOS-2 induction. The 3`-hydroxyl moiety seemed to reduce the activity, since kaempferole inhibited NOS-2 induction more strongly than quercetin. The 7 and 4`- hydroxyl groups may have affected inhibitory activity favourably, since apigenin was much more active than primuletin. In our model rutin, a flavonoid glycoside, was a very weak inhibitor of NOS-2 induction. Comparison of three-dimensional structures of strong and weak inhibitors reveals that a planar ring system in the flavonoid molecule is important to exhibit inhibitory activity of NOS-2 induction (*Fig. 4*). Our results are in line with those obtained on LPS-trated RAW 264.7 cells by Kim *et all*. (22).

What is the mechanism by which flavonoids inhibit the induction of NOS-2 in LPS-treated J774.2 cells? As oxygen free radicals were shown to contribute to NOS-2 induction in LPS-activated macrophages (23), one could argue that the effect of kaempferole and quercetin is due to widely recognized antioxidant properties of these compounds (17, 24). However this, cannot be a case, as in our model either extracellularly acting antioxidants (SOD, Catalase) or membrane-permeable scavengers [Mn(III)TBAP, Mn(III)TMPyP] (25) did not affect NOS-2 induction (data not shown). Moreover, kaempferole and quercetin are almost equipotent scavengers of superoxide anions (24), but they differ significantly in term of NOS-2 induction inhibition. Yet it should be noted, that flavonoids are not only potent scavengers of oxygen free radicals, but they may also inhibit NAD(P)H oxidase, a main source of endogenous free radicals (26). Thus, in our model we can not exclude the influence of flavonoids on free radical generation.

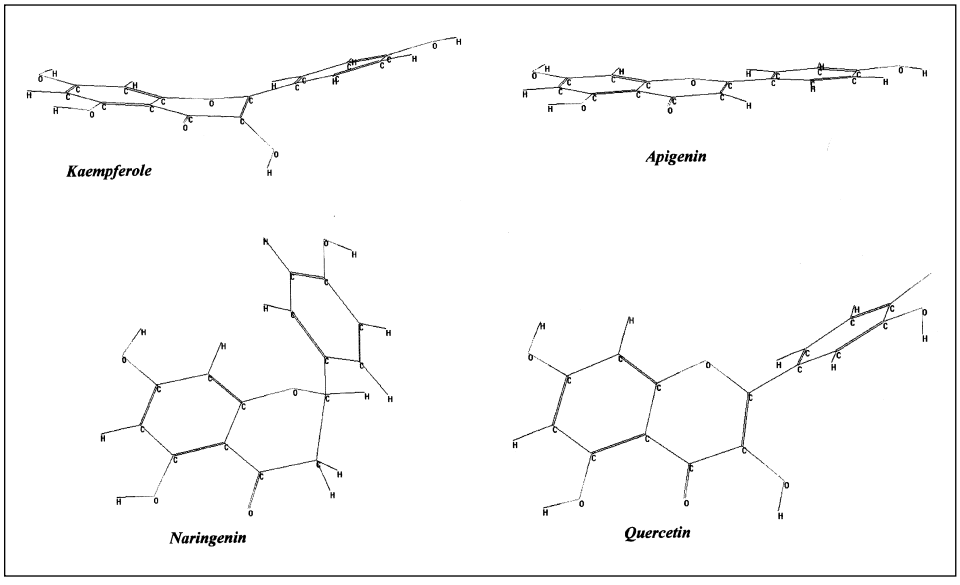


Fig 4. Three-dimensional structures of selected flavonoids, calculated with use of HyperChem 3.0 software. Strong inhibitors (kaempferole, apigenin) of NOS-2 induction in LPS-treated J774.2 cells possess more planar molecules than weak inhibitors (quercetin, naringenin).

In theory, there are several critical steps at which flavonoids may modulate cascade of molecular events leading to the expression of NOS-2 in LPS-treated macrophages. The response of the cells to LPS involves stimulation of membrane protein CD14 and toll like receptors (TLRs) with subsequent activation of downstream intracellular kinases and nuclear factors, such as nuclear factor κB (NF κB), which trigger transcription of NOS-2 gene (4, 27). It is demonstrated that flavonoids are inhibitors of protein tyrosine kinases (PTKs) (28, 29) and that is why they decrease cytokine secretion from LPS-treated macrophages (30). However, quercetin and kaempferole seem to be equally potent PTK inhibitors (14), so this mechanism hardly explains differences in their potencies of inhibition of NOS-2 induction.

Certain flavonoids were reported previously to inhibit protein kinase C, phospholipase A_2, phospholipase C and phosphodiesterases (14). Another possibility includes modulation of NOS-2 induction indirectly by inhibition of the cyclooxygenase and/or lipoxygenase pathways (31, 32). However, so far the contribution of particular mechanisms to NOS-2 induction in LPS-treated macrophages is not clear.

All pathways of of NOS-2 induction seem to converge in the activation of the essential transcription factor NF κB (4). Interestingly, some flavonoids were reported to inhibit NF κB activation through inhibition of IκB kinases (33-35). Does this mechanism contribute to the action of kaempferole and apigenin on NOS-2 induction in LPS-treated J774.2 cells? It remains to be investigated.

It is reported that flavonoids stimulate endothelial cells to release NO via increase of intracellular calcium level $[Ca^{++}]_i$ (12, 13). Indeed, infusion of flavonoids into coronary circulation of isolated guinea pig heart caused immediate vasodilatation. These responses were strongly inhibited by the L-NAME, non-selective NOS inihibitor, which also abolished response to bradykinin, standard NO-dependent vasodilator (19). Interestingly, in contrast with the infuence on NOS-2 induction, quercetin appeared to be a more potent endothelial NOS-3 simulator than kaempferol and apigenin. Apparently, flavonoids stimulate endothelial NOS-3 and inhibit NOS-2 induction in LPS-treated macrophages by different mechanisms.

We conclude that some flavonoids are inhibitors of NOS-2 induction due to the inhibition of NOS-2 gene transcription. At the same time, they may increase endothelial NOS-3 activity. The question arises whether flavonoids may prove beneficial in conditions in which regulatory, protective NOS-3 activity is overwhelmed by excessive NOS-2 induction, such as endotoxic shock or atherosclerosis. Importantly, flavonoids were recently demonstrated to protect mice from endotoxin lethality (36). Moreover, epidemiological studies show that the low incidence of coronary heart disease in the French and other Mediterranean populations, despite a diet rich in saturated fat, can be attributed to the high rate of polyphenol consumption derived from wine, tea or various plant foods (37, 38). We are tempted to speculate that some flavonoids may become parents of future drugs, which may become useful in the therapy of a variety of diseases associated with induction of the NOS-2 and suppression of the NOS-3.

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