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## Suppression of nitric oxide production in mouse macrophages by soybean flavonoids accumulated in response to nitroprusside and fungal elicitation

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### Abstract

**Background:** The anti-inflammatory properties of some flavonoids have been attributed to their ability to inhibit the production of NO by activated macrophages. Soybean cotyledons accumulate certain flavonoids following elicitation with an extract of the fungal pathogen *Diaporthe phaseolorum* f. sp. *meridionalis* (Dpm). Sodium nitroprusside (SNP), a nitric oxide donor, can substitute for Dpm in inducing flavonoid production. In this study, we investigated the effect of flavonoid-containing diffusates obtained from Dpm- and SNP-elicited soybean cotyledons on NO production by lipopolysaccharide (LPS)- and LPS plus interferon- $\gamma$  (IFN $\gamma$ )-activated murine macrophages.

**Results:** Significant inhibition of NO production, measured as nitrite formation, was observed when macrophages were activated in the presence of soybean diffusates from Dpm- or SNP-elicited cotyledons. This inhibition was dependent on the duration of exposure to the elicitor. Daidzein, genistein, luteolin and apigenin, the main flavonoids present in diffusates of elicited cotyledons, suppressed the NO production by LPS + IFN $\gamma$  activated macrophages in a concentration-dependent manner, with IC<sub>50</sub> values of 81.4  $\mu$ M, 34.5  $\mu$ M, 38.6  $\mu$ M and 10.4  $\mu$ M respectively. For macrophages activated with LPS alone, the IC<sub>50</sub> values were 40.0  $\mu$ M, 16.6  $\mu$ M, 10.4  $\mu$ M and 2.8  $\mu$ M, respectively. Western blot analysis showed that iNOS expression was not affected by daidzein, was reduced by genistein, and was abolished by apigenin, luteolin and Dpm- and SNP-soybean diffusates at concentrations that significantly inhibited NO production by activated macrophages.

**Conclusions:** These results suggest that the suppressive effect of flavonoids on iNOS expression could account for the potent inhibitory effect of Dpm- and SNP-diffusates on NO production by activated macrophages. Since the physiological concentration of flavonoids in plants is normally low, the treatment of soybean tissues with SNP may provide a simple method for substantially increasing the concentration of metabolites that are beneficial for the treatment of chronic inflammatory diseases associated with NO production.

## Background

Flavonoids are members of a class of natural pigments ubiquitous to plant cells and have a broad range of biological actions [1,2]. Various natural flavonoids are known to have anti-inflammatory activity in mammalian cells, and their action in inflammation has been attributed to their antioxidant activity, as well as to their ability to suppress NO production in macrophages [3-8].

NO synthesis by phagocytes depends on the expression of an NOS isoform (iNOS), which is induced by interferon- $\gamma$  (IFN $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and bacterial endotoxins, and is crucial for eliminating intracellular pathogens internalized by these cells [9,10]. However, the continuous elevated production of NO may account for several disorders associated with chronic inflammatory diseases [11,12]. Thus, natural flavonoids are very promising as therapeutic agents for the treatment of inflammation [13]. Several classes of natural flavonoids inhibit NO production by inflammatory cells such as activated peritoneal macrophages, RAW 264.7 cells and C6-astrocytes, *in vitro* [7,8,14-16]. The observation that pretreating mice with flavonoids suppressed the expression of proinflammatory molecules and reduced the lethality of LPS [17] indicates that natural flavonoids can also modulate the inflammatory process *in vivo*.

Many natural flavonoids have their biosynthesis activated in response to attack by pathogens [18]. The antibiotic activity of these secondary metabolites has been considered to have an important role in the protection of plants against microbe invasion [19]. Different classes of flavonoids are produced depending on the plant-microbe interaction [20]. The isoflavones daidzein and genistein, and the flavones apigenin and luteolin were recently identified as the main flavonoids accumulated in soybean cotyledons elicited with an extract of the phytopathogenic fungus, *Diaporthe phaseolorum* f. sp. *meridionalis* (Dpm), the causal agent of soybean stem canker disease [21]. An increase in L-citrulline production from L-arginine preceded *Diaporthe*-induced flavonoid biosynthesis in cotyledons, and pretreatment of cotyledons with NOS inhibitors reduced the flavonoid accumulation. Although the protein responsible for this activity has not yet been identified, these results suggested that soybean plants have a NOS enzymatic activity responsible for the production of NO that in turn acts as a signaling molecule for the activation of flavonoid biosynthesis. In fact, sodium nitroprusside (SNP), a nitric oxide (NO) donor, can substitute for Dpm in inducing flavonoid accumulation in soybean tissues [21].

Based on these findings, in the present study, we investigated the effects of flavonoid-containing diffusates obtained from elicited soybean cotyledons on NO pro-

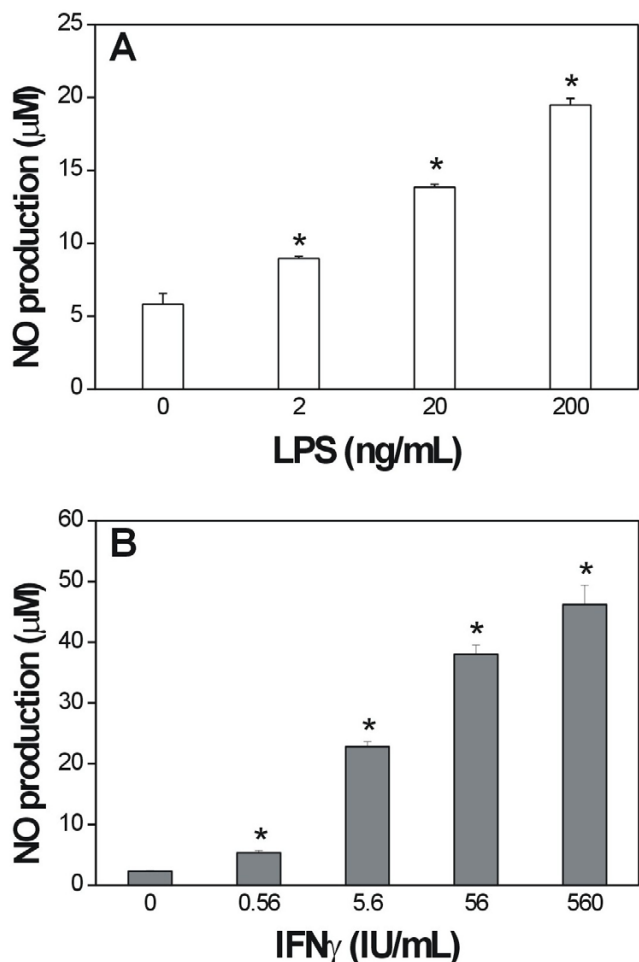
duction by stimulated peritoneal macrophages. The results show that flavonoids produced in response to Dpm or SNP can modulate iNOS expression and activity in macrophages to different extents.

## Results

To determine the best conditions for inducing NO production by mouse macrophages, adherent peritoneal cells pre-treated with thioglycollate medium were cultured for 48 h in the presence of increasing concentrations of bacterial LPS. As shown in Fig. 1, LPS stimulated NO production by murine macrophages in a concentration-dependent manner (Panel A). The combination of LPS (20 ng/mL) with different concentrations of IFN $\gamma$  resulted in a synergistic effect, which enhanced the amounts of NO released by macrophages compared to the endotoxin alone (Fig. 1B). Based on these results, we chose an LPS concentration of 20 ng/mL alone or in combination with 56 IU of IFN $\gamma$  /mL to induce NO production in subsequent experiments.

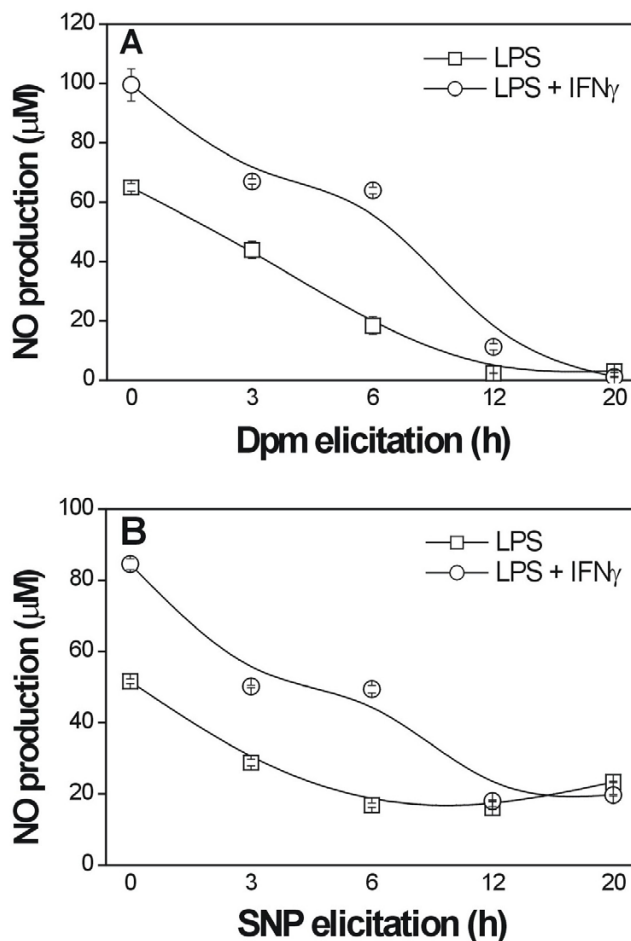
To examine the effect of soybean diffusates on NO production by LPS- or LPS + IFN $\gamma$ -stimulated peritoneal macrophages, the cells were treated with the inflammatory stimuli together with a 10-fold dilution of the plant diffusates, which were collected at different times after Dpm or SNP elicitation. As shown in Fig. 2, diffusates from Dpm- and SNP-elicited cotyledons (diluted 10-fold) had an inhibitory effect on NO production by LPS- or LPS + IFN $\gamma$ -activated macrophages that increased with the duration of elicitation. Significant inhibition of NO production was observed when murine macrophages were treated with diffusates collected as early as 6 h after exposure to the elicitors, and NO production was completely abolished by treating the cells with diffusates collected after 20 h (Fig. 2). The measurement of cell viability using the MTT assay showed that treatment of the macrophages with diffusates collected from soybean cotyledons elicited with Dpm or SNP did not cause macrophage death (data not shown).

Previous analysis has shown that daidzein, genistein, luteolin and apigenin are the main flavonoids present in Dpm- and SNP-elicited cotyledons [21]. According to these results, time course analysis of isoflavone production using HPLC with detection at 286 nm revealed that daidzein and genistein were already detected after 6 h of elicitation with Dpm (3 mg/L and 6 mg/L, respectively) or SNP (24 mg/L and 42 mg/L, respectively). Maximal production of these compounds was reached after 12 h of elicitation with Dpm (daidzein – 60 mg/L and genistein – 39 mg/L) and SNP (daidzein – 91 mg/L and genistein – 60 mg/L). The level of these isoflavones decreased or remained unchanged after 20 h of Dpm- or SNP-elicitation, respectively. The soybean diffusates have also



**Figure 1**  
Concentration-dependent production of NO by macrophages stimulated with LPS and IFN $\gamma$ . Macrophages were stimulated with LPS (A) or LPS (20 ng/mL) plus IFN $\gamma$  (B) for 48 h, after which the cells were harvested and the NO released was measured as nitrite using the Griess reagent. The columns represent the means  $\pm$  SE of three independent experiments, each done in quadruplicate. \* $P < 0.05$  (by Student's *t* test).

previously been analyzed for the presence of the flavones apigenin and luteolin since a spectral analysis showed a high absorbance at 350 nm, a wavelength typical of these compounds. Both elicitors induced a maximal accumulation of luteolin after 12 h (5.0 mg/L and 8.4 mg/L for Dpm- and SNP-elicitation, respectively), which then decreased after 20 h of incubation. Apigenin was induced mainly by Dpm and began to appear only after 12 h of elicitation (1.6 mg/L), increasing to 2.9 mg/L after 20 h, while SNP elicited the production of 1.2 mg of apigenin/L during this same period [21].

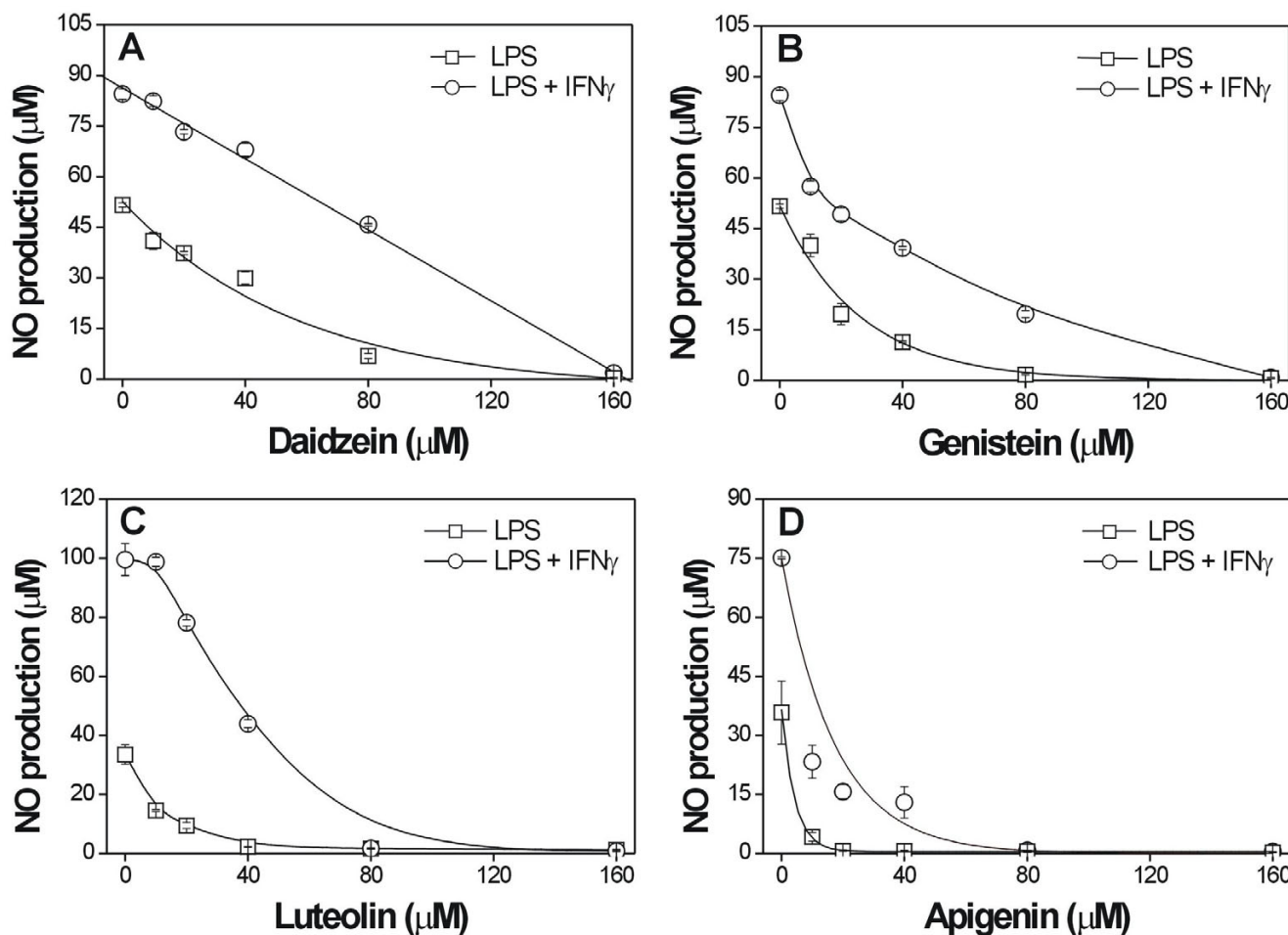


**Figure 2**  
Effect of diffusates from elicited cotyledons on NO production by stimulated macrophages. Cells were cultured for 48 h with LPS (20 ng/mL) (square symbols) or LPS (20 ng/mL) plus IFN $\gamma$  (56 IU/mL) (circle symbols) in the presence of plant diffusates collected at different times after Dpm (A) or SNP (B) inoculation. The concentrations of NO were expressed as nitrite. The points are the means  $\pm$  SE of three independent experiments, each done in quadruplicate.

Considering that the flavonoid produced in higher amounts in soybean diffusates was daidzein (91 mg/L after 12 h of SNP elicitation) and that these diffusates were diluted ten-fold for the macrophage treatments, they would contain no more than 9.1 mg/L of each identified flavonoid since these compounds have a similar molecular weight. Thus, in the present work, we examined the effect of the main flavonoids present in soybean diffusates on NO production by macrophages, when applied at 10  $\mu$ M to 160  $\mu$ M, which would be equivalent to 2.5 to 40 mg/L of daidzein, and thus represent a concentration range that included the amounts of the different

flavonoids found in the diffusates used to treat the macrophages. The results showed that, in the concentration range applied, all of the flavonoids inhibited NO production by macrophages, although to different extents (Fig. 3). Daidzein was the least potent, with an  $IC_{50}$  (the concentration causing 50% inhibition of NO production) of 40.0  $\mu\text{M}$  and 81.4  $\mu\text{M}$  in macrophages stimulated with LPS and LPS +  $IFN\gamma$ , respectively. Complete inhibition was observed only at 80  $\mu\text{M}$  and 160  $\mu\text{M}$  of daidzein in macrophages stimulated with LPS and LPS +  $IFN\gamma$ , respectively (Fig. 3A). Genistein inhibited NO production with an  $IC_{50}$  of 16.6  $\mu\text{M}$  and 34.5  $\mu\text{M}$  in macrophages stimu-

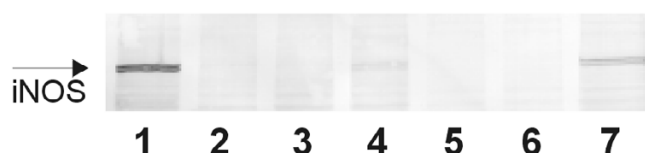
lated with LPS and LPS +  $IFN\gamma$ , respectively (Fig. 3B). The  $IC_{50}$  for luteolin was 10.4  $\mu\text{M}$  and 38.6  $\mu\text{M}$  for stimulation with LPS and LPS +  $IFN\gamma$ , respectively (Fig. 3C). Apigenin was the most potent inhibitor and significantly reduced NO production by activated macrophages at concentrations as low as 10  $\mu\text{M}$  (88% and 69% inhibition in LPS- and LPS +  $IFN\gamma$ -activated macrophages, respectively) (Fig. 3D). The estimated  $IC_{50}$  for apigenin was 2.8  $\mu\text{M}$  and 10.4  $\mu\text{M}$ , respectively. In the concentration range used here, none of the flavonoids were toxic to the macrophages, as measured by MTT assays (data not shown).



**Figure 3**

Dose-dependent effect of the isoflavones daidzein (A) and genistein (B) and the flavones luteolin (C) and apigenin (D) on NO production by stimulated macrophages. The cells were cultured for 48 h with LPS (20 ng/mL) (square symbols) or LPS (20 ng/mL) plus  $IFN\gamma$  (56 IU/mL) (circle symbols) in the presence of the indicated concentrations of isoflavones and flavones. The amount of NO released into the culture supernatants was expressed as nitrite. The points are the means  $\pm$  SE of three independent experiments, each done in quadruplicate.

To determine whether the reduced NO production by macrophages exposed to soybean diffusates or to individual flavonoids was due to the inhibition of iNOS expression, crude extracts obtained from treated cells were analyzed by western blotting. In these experiments macrophages were stimulated for 12 h with LPS + IFN $\gamma$  alone or in combination with 80  $\mu$ M apigenin or luteolin, 160  $\mu$ M daidzein or genistein or 1/10 dilution of Dpm- or SNP-elicited diffusates. As shown in Figure 4, iNOS expression was prevented when macrophages were stimulated with LPS + IFN $\gamma$  in the presence of soybean diffusates collected from cotyledons elicited with Dpm or SNP for 20 h. Figure 4 also shows that, iNOS expression was inhibited when macrophages were activated in the presence of the flavonoids apigenin or luteolin. The isoflavones genistein and daidzein were less effective in preventing iNOS expression. The former slightly reduced the enzyme expression, whereas daidzein had no effect at concentrations that completely abolished NO production by activated macrophages (Fig. 3).



**Figure 4**  
Effect of soybean diffusates and flavonoids on iNOS expression in macrophages. Western blots were done using macrophage extracts obtained from cells activated with 20 ng/mL LPS plus 56 IU/mL IFN $\gamma$  alone (Lane 1) or together with diffusates from soybean elicited with Dpm (Lane 2) or SNP (Lane 3) or with the flavonoids genistein (Lane 4), apigenin (Lane 5), luteolin (Lane 6) or daidzein (Lane 7). The arrow indicates a protein of ~130 kDa detected by the anti-mac NOS antibody.

## Discussion

Flavonoids with antimicrobial activity are produced by plants in response to pathogen attack and are designed to prevent pathogen replication and spreading. Such responses can be induced either by elicitor components extracted from the pathogen or by abiotic elicitors. In soybean, an elicitor preparation of the phytopathogenic fungus *Diaporthe phaseolorum* f. sp. *meridionalis* (Dpm) induces the production of flavonoids and this response can be mimicked by the abiotic elicitor sodium nitroprusside [21].

Our results show that diffusates from soybean cotyledons elicited with a Dpm extract, as well as with SNP, adversely affected NO production induced by LPS or LPS + IFN $\gamma$  in

mouse peritoneal macrophages. This inhibitory activity of the soybean diffusates resulted from the inhibition of iNOS expression and/or activity. Although these stimuli did not cause macrophage death, we cannot rule out the possibility that such treatments may have other adverse side effects, and should be investigated further in studies *in vivo*.

As shown elsewhere [21], Dpm and SNP diffusates contain mainly the phenylpropanoid derivatives daidzein, genistein, apigenin and luteolin, whose content increases with the duration of exposition of soybean cotyledons to the elicitor. To determine whether these main flavonoids present in Dpm- and SNP-elicited soybean diffusates could affect NO release by macrophages, mouse adherent peritoneal cells were treated with different concentrations of commercial preparations of these compounds during exposure to the inflammatory stimuli. The main flavonoids identified in the diffusates of elicited soybean cotyledons were able to inhibit NO production by activated macrophages in a dose-dependent manner. Based on the IC<sub>50</sub> values obtained with moderate (LPS) and intense (LPS + IFN $\gamma$ ) inflammatory stimuli, the order of potency for the inhibition of NO production by peritoneal macrophages was apigenin > luteolin > genistein > daidzein.

Western blot analysis showed that the inhibitory effect of the Dpm and SNP diffusates on NO production resulted mainly from the inhibition of macrophage iNOS expression by the flavonoids (Fig. 4). Accordingly, apigenin and luteolin prevented, genistein reduced and daidzein did not change iNOS expression in macrophages when tested at concentrations that significantly reduced NO production. The inhibitory effect of daidzein on NO production by macrophages may result from a direct inhibitory effect of this flavonoid in iNOS enzyme activity. The effectiveness of the inhibitory action of these compounds on NO production correlated positively with the extent to which iNOS expression was prevented in macrophages. Thus, the flavonoids apigenin and luteolin were more effective than the isoflavones genistein and daidzein in inhibiting NO production by activated peritoneal macrophages (Fig. 3), probably because of their ability to prevent iNOS enzyme expression. Kim and coworkers [3] showed that the inhibitory activity of natural flavonoids on NO production in LPS-activated RAW 264.7 macrophage cells was related to their chemical structure, i.e., flavonoids with a C-2,3 double bond, such as wongonin and luteolin, were able to inhibit strongly NO production through their ability to reduce iNOS expression. Liang and coworkers [22] showed that the inhibitory effect of apigenin and related flavonoids on NO production by LPS-activated RAW 264.7 cells was due to their ability to suppress the activation of nuclear factor kappa B (NF- $\kappa$ B), a transcription factor involved in regulating the expression

of iNOS, and other inflammatory mediators. Other phytochemicals, such as resveratrol and avicins, also inhibit NO production by suppressing NF- $\kappa$ B activation [23,24]. Based on these studies, the potent inhibitory effect of the flavones used here could be attributed to their ability in suppress the transcriptional activation of iNOS in peritoneal macrophages.

The observation that iNOS expression in macrophages was also prevented when the cells were treated with diffusates elicited with Dpm or SNP suggests that the flavones, mainly apigenin, present in such diffusates could account for most of the inhibitory action of the diffusates on NO production in these cells. In agreement with this, time-course analyses of the accumulation of flavonoids showed that daidzein, genistein and luteolin began to appear after 6 h and reached peak levels after 12 h of Dpm-elicitation, whereas apigenin appeared only after 12 h and increased for up to 20 h after elicitation [21]. Indeed, the amount of flavonoids present in each diffusate correlated well with their inhibitory effect on NO production by macrophages. Thus, diffusates (10-fold diluted) collected 6 h after exposure to Dpm extract caused only partial inhibition (35.7%) of NO production by macrophages in the presence of strong inflammatory stimuli (LPS + IFN $\gamma$ ), and such diffusates contained 0.3 mg/L daidzein, 0.6 mg/L genistein; 0.08 mg/L luteolin and 0.001 mg/L apigenin, concentrations that were considerably below the IC<sub>50</sub> for the inhibition of NO production by these flavonoids (20.7 mg/L, 9.3 mg/L, 11 mg/L and 2.8 mg/L, respectively). Increased amounts of these flavonoids were present in 10-fold diluted 12 h Dpm diffusates (5.9 mg/L daidzein, 3.9 mg/L genistein, 0.5 mg/L luteolin and 0.16 mg/L apigenin) and, although the concentration of each flavonoid was below the corresponding IC<sub>50</sub>, the Dpm diffusates still showed a very potent inhibitory activity (88.6%) on NO production, suggesting a synergistic interaction between the flavonoids. NO production was abolished with 20 h Dpm diffusates. Although this extract contains lower amounts of daidzein (2.14 mg/L), genistein (0.9 mg/L) and luteolin (0.14 mg/L) its apigenin content (0.3 mg/L) is considerably increased compared with that of 12 h diffusates. These results suggest that apigenin could account for the potent inhibitory effect of 20 h Dpm-diffusates on NO production by activated macrophages, through its ability in suppress iNOS expression (Fig. 4).

SNP instead of Dpm could also substantially increase the inhibitory effect of soybean diffusates on iNOS expression and activity in macrophages. Although the 10-fold diluted 20 h SNP diffusates contained lower levels of apigenin (0.12 mg/L) than the Dpm diffusates (0.3 mg/L), the contents of daidzein, genistein and luteolin (10.2 mg/L, 4.2 mg/L and 0.4 mg/L, respectively) was much higher than

that of the Dpm diffusates (2.1 mg/L, 0.9 mg/L and 0.14 mg/L, respectively). Thus, the inhibitory effect of SNP diffusates on NO production by murine macrophages may result from a synergistic interaction of the different flavonoids in inhibiting iNOS activity and expression. Since the physiological concentration of flavonoids in plants is usually low [2], these results suggest that SNP may be used to increase the production of metabolites that could serve as therapeutic agents for the treatment of several types of inflammatory diseases.

Although the results obtained here may not explain the control of NO production by flavonoids during the elicitation of soybean cotyledons, they do provide information for future experiments that may help in understanding the molecular interactions between flavonoids and NO during plant responses to microbial invasion. NO is an upstream element in the signal cascade that leads to the synthesis of soybean flavonoids in response to Dpm-elicitation [21]. This finding supports previous observations that NO can activate defense genes associated with initial enzymes of the phenylpropanoid pathway [25,26]. Once produced, flavonoids with antimicrobial activity would protect the plant against invading pathogens. However, an excess of NO can lead to tissue damage so that flavones produced later could prevent the deleterious effects of excessive NO through their excellent ability to scavenge radicals [2]. In animal cells, flavonoids can modulate signaling pathway components such as NF- $\kappa$ B involved in NO production prior to affecting iNOS expression [27]. Although no counterparts for these factors have been described in plants, the control of the transcriptional activation of an NOS-like enzyme by flavonoids could down regulate NO synthesis, thereby preventing an excess of nitrogen radical generation during plant-microbe interactions.

## Conclusions

Flavonoid-containing diffusates obtained from Dpm- and SNP-elicited soybean cotyledons inhibited NO production induced by LPS or LPS + IFN $\gamma$  in mouse peritoneal macrophages. The inhibitory activity of the soybean diffusates on NO production by macrophages resulted from the suppressive effect of its flavonoids content on iNOS activity and expression. SNP-elicitation could be used to increase the production of soybean flavonoids of medical interest for the treatment of inflammatory diseases associated with NO production.

## Methods

### Animals

Female BALB/c mice (1–4 weeks old) were obtained from the Central Animal House Service (CEMIB) at UNICAMP. The mice were maintained in a temperature-controlled room in the Department of Microbiology and

Immunology and received water and food *ad libitum* throughout the study.

### Reagents

The standard flavonoids luteolin, apigenin, daidzein, genistein and other reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The reagents used in electrophoresis were purchased from Bio-Rad (Hercules, CA, USA).

### Plant material

Soybean (*Glycine max* [L.] Merr.) seeds of the cultivar IAC-18, resistant to the fungus *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. f. sp. *meridionalis* (Dpm), were grown in vermiculite at room temperature in a green house. Cotyledons detached from 7–8-day-old seedlings were used for the elicitation assays.

### Preparation of the elicitor

Elicitor from Dpm (strain 8498) was obtained by autoclaving aqueous spore suspensions of 30–40-day-old cultures grown in PDA media in the dark at room temperature. The autoclaved suspension was centrifuged at 10,000 g for 6 min and the pellet was discarded. Oligosaccharides extracted from the cell wall of Dpm spores, which are compounds known to elicit defence response in plant tissues [28], were quantified in the supernatant as total carbohydrates by the phenol-sulfuric procedure using glucose as standard. Oligosaccharides from Dpm (0.4 mg/mL) and an aqueous solution of sodium nitroprusside (SNP, 10 mM) were used for soybean cotyledon elicitation.

### Elicitation assay

The plant diffusates were prepared using the soybean cotyledon assay in which a small section (i.d. = 1.0 cm) was removed from the adaxial surface of each cotyledon and the wounded surface was then treated with 50  $\mu$ L of elicitor (Dpm or SNP). The cotyledons were kept in the dark at 26°C for different periods of time, after which the diffusates were collected and diluted ten fold for use in the activation of macrophages. The efficiency in eliciting flavonoid production was evaluated by HPLC analysis, as previously described [21].

### Macrophage culture

Macrophages were harvested with 3 mL of RPMI medium from peritoneal cavities of naive or stimulated (1–3 mL of 3% thioglycollate medium; Gibco, Gaithersburg, MD; 4 days before) mice. The cells were centrifuged and adjusted to  $1 \times 10^6$ /mL in RPMI 1640 medium supplemented with 12.5 mM HEPES, 2 g of sodium bicarbonate/L, 2 mM L-glutamine, 50  $\mu$ g of gentamicin/mL and 10% fetal bovine serum (complete medium). The cell suspensions were seeded onto 96-well flat-bottomed plates (Corning Cor-

poration; Cambridge, MA, USA) at a density of  $2 \times 10^5$ /200  $\mu$ L/well and then incubated for 2 h in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C to allow the cells to adhere. Adherent cells (95% macrophages) were washed three times with PBS (pH 7.2), and incubated for 48 h with either RPMI medium alone or medium containing the indicated concentrations of LPS and/or IFN $\gamma$ . Different amounts of standard flavonoids or soybean diffusates were added to the cultures simultaneously with the stimuli.

### Measurement of nitrite concentration

NO released into the supernatants of mouse macrophages was determined by the standard Griess reaction by adding 50  $\mu$ L of test solution to 96-well flat-bottomed plates containing 50  $\mu$ L of Griess reagent [1% sulfanilamide/0.1% N-(1-naphthyl)ethylenediamine dihydrochloride/2.5% H<sub>3</sub>PO<sub>4</sub>]. The samples were assayed in quadruplicate. After 15 min at room temperature, the absorbance of each well was measured in a Multiskan MS microplate reader (Lab-systems Oy, Helsinki, Finland) at 540 nm and the nitrite concentration was determined from a standard curve of sodium nitrite. The results were expressed as  $\mu$ M nitrite/ $10^6$  cells. Statistical analysis was done using Student's *t* test, with a value of *P* < 0.05 indicating significance.

### iNOS detection by western blotting

Peritoneal macrophage monolayers were prepared as described above, except the cells were seeded onto plastic Petri dishes at a density of  $1 \times 10^7$  cells/5 mL/dish (Corning). After a 2 h incubation, the non-adherent cells were removed by washing and the macrophages were then treated with LPS + IFN $\gamma$  in the absence or presence of soybean diffusates or flavonoids, as indicated in the Results. The plates were incubated for an additional 12 h, under the same conditions as described above. To remove the stimuli, the plastic dishes were washed three times with PBS, and the cells then lysed with 1 mL of sample buffer (125 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue and 1%  $\beta$ -mercaptoethanol). The lysates were boiled for 3 min and the debris was removed by centrifugation at 10,000  $\times$  g (Eppendorf Micro Centrifuge 5415 C, Brinkmann Instruments, Inc., NY, USA) and the supernatant was used for western blotting.

The polypeptides in macrophage samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to [29], using a 7% acrylamide slab gel with a 3% acrylamide stacking gel, in a Mini-Protean® II Dual Slab Cell (Bio-Rad). The current was set at 25 mA and the gels were run for 2 h. After electrophoresis, the gels were blotted onto 0.45  $\mu$ m nitrocellulose membranes (Schleicher & Schüll, Inc., Keene, NH, USA) for 2 h at 180 mA [30]. The membranes were then blocked by incubation for 1 h with 5% non-fat powdered milk in Tris-buffered saline

(TBS: 20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.05% Tween 20 (TBS-Tween), and then rinsed three times with TBS-Tween. The protein blots were probed with mouse IgG<sub>2a</sub> anti-macNOS at 0.5 µg.mL<sup>-1</sup> (Transduction Laboratories, Lexington, KY, USA). After overnight incubation at 4°C, the blots were washed six times with TBS-Tween (10 min each) at room temperature followed by treatment overnight at 4°C with an anti-mouse rabbit IgG-horseradish peroxidase conjugate in TBS-Tween. The blots were then washed six times with TBS (10 min each) at room temperature and the colour reaction was developed using 0.6 mg 3,3'-diaminobenzidine/mL (Sigma) in 50 mM Tris-HCl, pH 7.4, and 0.03% H<sub>2</sub>O<sub>2</sub>.

### Authors' contributions

LSS, PUS and DLG carried out the experiments with macrophage cells, EES carried out the western blotting, WMSCT supervised the work of LSS, PUS and DLG and participated in drafting the manuscript, LVM prepared the soybean diffusates and participated in drafting the manuscript, and IS conceived the study, participated in its design and coordination, and drafted the manuscript. All authors read and approved the final manuscript.

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