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INHIBITION OF CONSTITUTIVE ENDOTHELIAL NO-SYNTHASE ACTIVITY BY TANNIN AND QUERCETIN

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Abstract—The effect of natural polyphenols on three isoforms of NO-synthase was investigated. Among the compounds tested, tannin was the most potent, inhibiting endothelial constitutive NO synthase (eNOS) with an IC_{50} of 2.2 μM. Other NOS isoforms (i.e. neuronal constitutive NOS and smooth muscle inducible NOS) were also inhibited but at much higher concentrations (selectivity ratio of approx. 20–30). Quercetin was also an effective but less potent inhibitor of eNOS (IC_{50} = 220 μM). The kinetics of tannin inhibition were investigated to gather information on the mechanism of action. Tannin did not interfere with the interaction of the enzyme with the co-substrates L-arginine and NADPH nor with the cofactor tetrahydrobiopterin. The inhibition level was also independent of free Ca^{2+} concentration as well as of the presence of high exogenous calmodulin concentrations.

Key words: tannin; quercetin; inhibition; NO-synthase; EDRF; constitutive; endothelial; vasodilatation

Plant phenols, in particular bioflavonoids, exert several physiological actions on animals. The most studied effects are those related to the vascular system. It was observed long ago that various natural flavonoids increase the resistance of normal capillaries to trauma [1] and that after injection they can modulate blood pressure. The phenolic compound tannin, a major component of the dust in cotton mills [2], is possibly involved in the genesis of byosinosis, an occupational lung disease affecting mill workers and characterized by chest tightness and cough [3]. More recently, *in vitro* studies have been carried out to investigate a possible direct effect of tannin and related compounds on the vasculature. Micromolar concentrations of tannin were shown to display complex effects on the contractile behaviour of pulmonary arterial rings of the rabbit [4]. Tannin caused a concentration-dependent contraction of the resting vessels, while it relaxed rings precontracted by norepinephrine [4]. This dual mode of action was shown to be dependent on the concomitant release of both relaxing and contracting factors from the endothelial cell layer (possibly in the form of NO and thromboxane A_2, respectively). Based on encouraging studies of a possible protective role of moderate alcohol intake (in particular red wine) on the incidence of coronary heart disease [5], Fitzpatrick *et al.* [6] recently investigated the effects of various components of grapes on rat aortic rings. It was found that grape skin extracts relaxed rings precontracted with phenylephrine and that this action was dependent on the presence of an intact endothelium. Similar results were obtained with tannin and quercetin, two major components of red grape skin. Since the level of cGMP in the vascular tissue was significantly increased by the treatment and, in addition, the effect was obliterated by selective inhibitors of NO-synthase (such as N^G-nitro-L-arginine and N^G-monomethyl-L-arginine), it was speculated that tannin and quercetin might interfere with the NO-cGMP† pathway [6].

In this study we investigated whether these bioflavonoids display a direct effect on the activity of endothelial constitutive NO-synthase, the enzyme responsible for the physiological production of the relaxing factor NO in the vasculature. Tannin was found to be a potent and fairly selective inhibitor of this enzyme. The mechanism of action of the compound was not related to substrate binding but rather to the physiological regulation by the Ca^{2+}–calmodulin complex.

MATERIALS AND METHODS

Materials. Tannin and the other plant phenols were obtained from Fluka (Buochs, Switzerland). Tetrahydrobiopterine, NADPH and all the protease inhibitors were from Sigma. Recombinant calmodulin was a gift from Dr E. Carafoli (ETH, Zurich, Switzerland). [^{3}H]Arginine was from Amersham (69 Ci/mmol). CGS 9343 was supplied by the Chemistry Department of Ciba-Geigy Ltd (Basle, Switzerland).

Endothelial NOS (eNOS) preparation. Bovine aortic endothelial cells were cultured in Petri dishes until confluent, rinsed with PBS, scraped and washed in PBS and then resupended and homogenized in a medium composed of 50 mM Tris–Cl, pH 7.4, 0.1 mM EDTA, 0.1 EGTA, 0.1% 2-mercapto-ethanol, 1 mM phenylmethylsulphonyl fluoride, 2 μM leupeptine, and 1 μM pepstatine A (Buffer A), basically as described in Ref. 7. Cells from passages 6–8 were utilized. After homogenization the

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† Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether *N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; L-NAME, N^G nitro-L-arginine methyl ester; NOS, NO synthase.

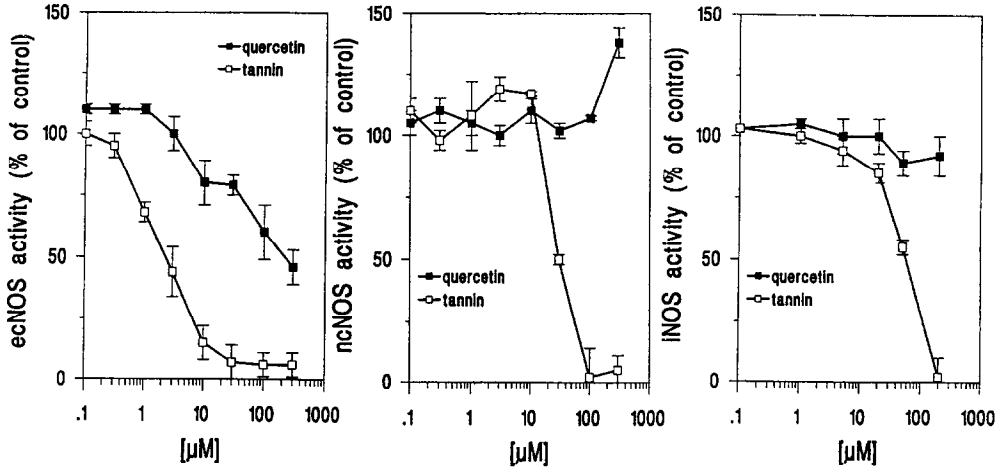

Fig. 1. Effect of tannin and quercetin on NOS activity. The effect of tannin and quercetin on the NOS activity of the various enzyme preparations was studied by measuring the oxidation rate of L-arginine to L-citrulline. Free Ca^{2+} concentration was 10 μM. The data are means ± SEM, N = 4. Endothelial constitutive NOS (ecNOS); neuronal constitutive NOS (ncNOS); smooth muscle inducible NOS (iNOS).

particulate fraction was obtained by centrifugation at 100,000 *g* for 30 min, resuspended and stored in Buffer A at −70° without any appreciable loss of eNOS activity for several weeks. For some experiments, endogenous calmodulin was extracted as follows: the particulate fraction (approx. 0.1 mg/mL) was incubated for 10 min in Buffer A supplemented with the calmodulin antagonist CGS 9343 (200 μM). After centrifugation the extraction was repeated in the presence of 100 μM CGS 9343. The extracted particulate fraction was resuspended in Buffer A and used in the assays.

Neuronal NOS (nNOS) preparation. The procedure originally described by Bredt and Snyder [8] for rat was applied to bovine brain. The tissue was homogenized in a buffer composed of 50 mM Tris–Cl, pH 7.4, 1 mM EDTA, 10 μg/mL antipain, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 10 μg/mL chymostatin and 100 μg/mL phenylmethylsulphonyl fluoride at 4°. The homogenate was centrifuged at 20,000 *g* for 15 min and the supernatant was aliquoted and frozen at −70°. nNOS activity was maintained for several weeks after preparation.

Vascular smooth muscle inducible NOS (iNOS). The expression of iNOS was induced as described in Ref. 9. Rat aortic vascular smooth muscle cells were cultured to confluence in Dulbecco's MEM supplemented with 10% fetal calf serum. The cells were then rinsed and incubated for 12 hr in Dulbecco's MEM in the absence of serum. Interleukin-1β was then added at a concentration of 30 ng/mL and the cells were incubated for an additional 12 hr. Thereafter the cells were rinsed in PBS, scraped and homogenized in Buffer A. The supernatant obtained after centrifugation at 20,000 *g* for 15 min was frozen at −70° and used in the assays.

Measurement of NOS activity. The conversion of radioactive labelled L-arginine to L-citrulline by the action of the various NOS preparations was measured basically according to Bredt and Snyder [8] as modified by Wolff and Datto [10]. The standard reaction medium contained 50 mM HEPES, pH 7.4, 2 mM EGTA and various amounts of $CaCl_2$ in order to obtain the required free Ca^{2+} concentration, 1 mM dithiothreitol, 1 mM NADPH, 10 μM tetrahydrobiopterine, and 0.3 μM calmodulin. A portion of the various NOS preparations and, when required, a specific concentration of the bioflavonoids dissolved in water were added to the reaction medium. After 10 min preincubation, the reaction was initiated at room temperature by the addition of L-arginine (normally 1 μM) supplemented with [^{3}H]arginine (2 μCi/mL reaction medium). After various time intervals (the reactions were quasi-linear for longer than 1 hr), the reaction was stopped by mixing with the weak cationic exchange resin AG 50W-X8 (10 g/100 mL in 20 mM morpholino ethanesulfonic acid (MES), pH 5.5, 2 mM EDTA). After 30 min mixing, the resin was sedimented and aliquots of the supernatant containing L-citrulline were mixed with scintillation fluid and counted (1250 Mikrobeta, Wallach, Zurich, Switzerland). Each data point was determined at least in triplicate. The overall procedure was adapted to be carried out in the 48-well millititre format. The results are given as means ± SEM.

RESULTS

The effect of tannin and other related bioflavonoids on the activity of eNOS was tested. Figure 1A shows the potent concentration-dependent inhibition of enzyme activity by tannin: a virtually complete inhibition could be obtained by concentrations above 10 μM with an IC_{50} value of approx. 2 μM. Quercetin also displayed inhibitory properties but with a much reduced potency (IC_{50} = 220 μM). Several other related compounds were tested, but no inhibition was detected up to a concentration of 300 μM (see Table 1).

Table 1. Effect of various plant phenols on the eNOS activity

Phenol	IC_{50} (μM)
Tannin	2.2 ± 0.7
Quercetin	220 ± 80
Rutin	>300*
Hesperidin	>300*
Catechin	>300*
Tricine	>300*

L-citrulline formation by the bovine eNOS preparation was determined in the presence of saturating calmodulin and Ca^{2+} concentration. The enzyme was preincubated with various amounts of bioflavonoids for 10 min. The data are means ± SEM, N = 4.

* No inhibition was observed at the highest concentration tested (300 μM).

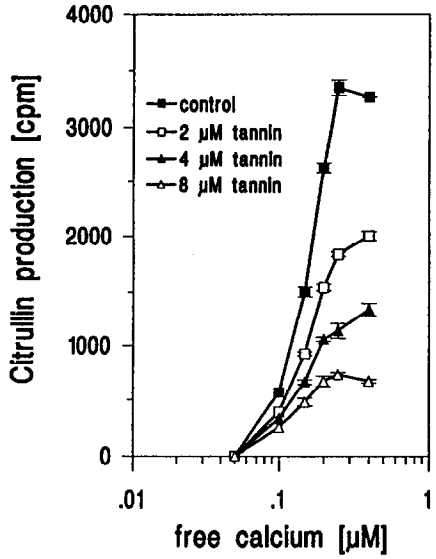

Fig. 3. Ca^{2+}-dependency of tannin inhibition of eNOS. The effect of tannin on the rate of L-arginine oxidation to L-citrulline by the eNOS preparation was studied at various free Ca^{2+} concentrations. The data represent the amount of citrulline produced in 20 min and are means ± SEM, N = 5.

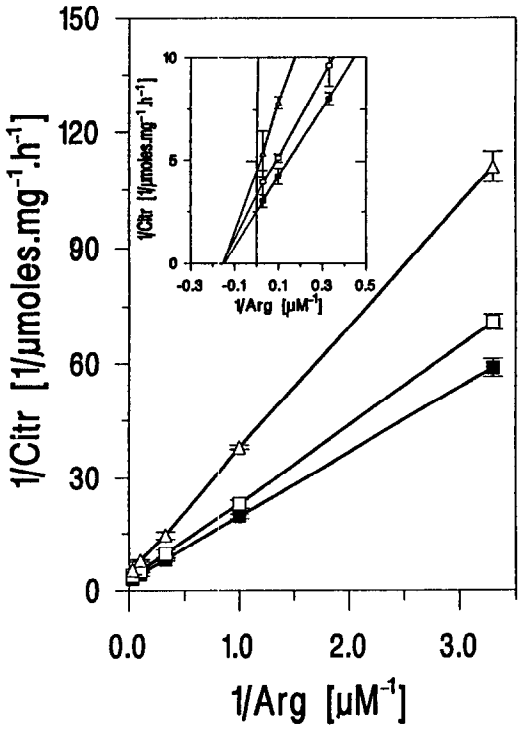

Fig. 2. Kinetic analysis of tannin inhibition of eNOS. The activity of the eNOS preparation was measured in the presence of 10 μM free Ca^{2+} and various concentrations of the substrate L-arginine and the effect of tannin thereupon was studied. The data are represented as a double reciprocal plot of the rate of L-citrulline production as a function of L-arginine concentration. The data are means ± SEM, N = 4. The insert represents a magnification of the region close to the interception of the axis.

The effects of tannin and quercetin were also tested on other known NOS isoforms. Figure 1B shows the concentration-dependent inhibition by tannin of brain nNOS activity. The compound was considerably less potent on the brain isoform than on endothelial cNOS (IC_{50} values of approx. 30 μM and 2 μM, respectively). No inhibitory action of quercetin could be detected. On the contrary, a small but significant stimulation of nNOS activity was observed at the highest concentration tested (300 μM) (see Fig. 1B). Interleukin-1β was used to induce the expression of the iNOS isoform in vascular smooth muscle cells in culture. The activity of the iNOS was completely independent of Ca^{2+} concentration between 0.05 and 100 μM (not shown). The action of the inhibitors on iNOS activity is presented in Fig. 1C. No effect was observed with quercetin. Tannin, however, exerted a strong inhibition, but the IC_{50} value was above 50 μM.

The inhibitory effect of tannin was reversible. The membrane preparation from bovine endothelial cells was incubated at room temperature for 30 min in the presence of 20 μM tannin (this concentration would normally produce a complete inhibition of eNOS). Thereafter the suspension was diluted 20-fold into the reaction mixture and the cNOS activity measured (final tannin concentration after dilution was 0.5 μM). The residual enzyme activity was identical to that of the controls, thus showing that tannin inhibition could be reversed simply by dilution.

The kinetic properties of NOS inhibition by tannin were investigated in further detail. Figure 2 shows a Lineweaver–Burk analysis of the activity of eNOS as a function of various concentrations of the substrate L-arginine. The plot shows a typical non-competitive mechanism of action of tannin. The degree of inhibition was identical when measured

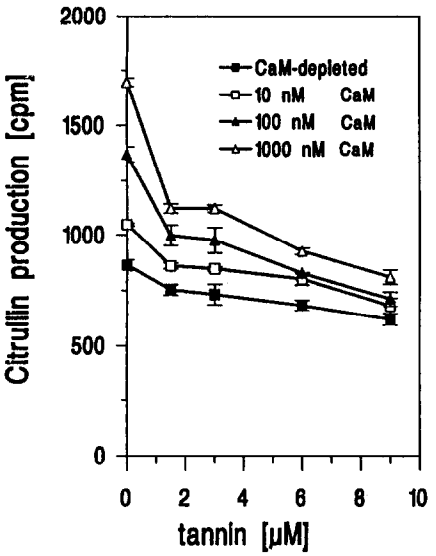

Fig. 4. Effect of tannin on the calmodulin-stimulation of extracted eNOS preparation. Endogenous calmodulin was extracted from the membrane preparation of eNOS by incubating with 100 μM of the calmodulin antagonist CGS 9443 for 30 min at room temperature. After centrifugation, the extraction procedure was repeated once more and the final pellet was used for NOS measurements. Inhibition by tannin was determined in the presence of various amounts of exogenous calmodulin. The data represent the amount of citrullin produced over a period of 20 min and are means ± SEM, N = 4.

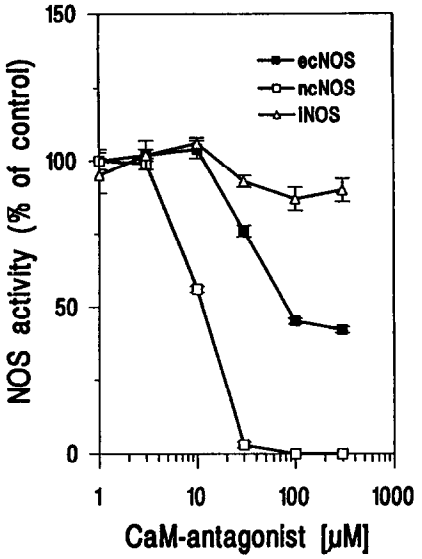

Fig. 5. Effect of the calmodulin antagonist CGS 9443 on the various NOS isoforms. Crude preparations of endothelial constitutive NOS (ecNOS), neuronal constitutive NOS (ncNOS) and smooth muscle inducible NOS (iNOS) were incubated for 10 min in the presence of various concentrations of the calmodulin antagonist CGS 9443 and NOS activity was then measured. Data are means ± SEM, N = 4.

over a wide range of substrate concentrations. The maximal velocity of the enzyme was reduced by tannin while the affinity for L-arginine remained practically unaltered. The K_m (L-arginine) value obtained from the double reciprocal plot was approx. 6–7 μM (see insert of Fig. 2), i.e. very close to the value of 2–3 μM previously reported for eNOS [7].

NADPH, together with O_2, is a co-substrate for the oxidation of L-arginine to L-citrulline by the NOS reaction. In a set of experiments the inhibition of eNOS by tannin was tested in the presence of various concentrations of NADPH ranging from 0.1 to 10 mM. The capability of tannin (2 μM) to inhibit L-citrulline formation was found to be virtually identical over a wide range of NADPH concentrations (not shown). Neither was a significant difference in tannin inhibition observed when the reaction was performed in the presence of various concentrations of tetrahydrobiopterine (from 0.1 to 100 μM).

In another set of experiments the effect of tannin on the Ca^{2+}–calmodulin dependence of the various NOS preparations was investigated. eNOS activity was found to be absolutely dependent on the free Ca^{2+} concentration in the medium: an EC_{50} of approx. 0.15–0.2 μM Ca^{2+} was obtained (see Fig. 3). The Ca^{2+} dependency curve was very steep; significant activity was detectable at free Ca^{2+} concentrations above 0.1 μM and was already maximal at concentrations above 0.3 μM. Tannin did not change the Ca^{2+} dependency of the enzyme and inhibited eNOS activity to the same extent at all free Ca^{2+} concentrations tested (see Fig. 3). As previously reported by Forstermann *et al.* [11], crude preparations of eNOS show no dependence on calmodulin since endogenous calmodulin associated with the membranes is sufficient for maximal activity. Also, our eNOS preparation was not dependent on calmodulin, and addition of large amounts (up to 3 μM) of exogenous calmodulin could not reverse tannin inhibition (not shown). We attempted to remove endogenously bound calmodulin by extracting the endothelial membrane fraction containing eNOS activity with 2 mM EGTA. The procedure, however, was not sufficiently effective. Better removal of endogenous calmodulin was achieved by extracting the membrane fraction twice (see Materials and Methods) in the presence of 100 μM CGS 9343—a potent and selective calmodulin antagonist [12]. After extraction, eNOS activity became partially dependent on the addition of exogenous calmodulin (Fig. 4). In the absence of exogenous calmodulin, however, a residual activity of approx. 40–50% of maximal activity was still observed. Interestingly, this residual activity was still completely Ca^{2+} dependent and fully inhibited by 10 μM L-NAME (not shown). Tannin, however, was less effective on residual activity, whereas it strongly inhibited stimulability by exogenous calmodulin (see Fig. 4). The results suggest that a portion of eNOS activity after extraction of endogenous calmodulin became more resistant to tannin inhibition. Additional experiments were carried out to clarify

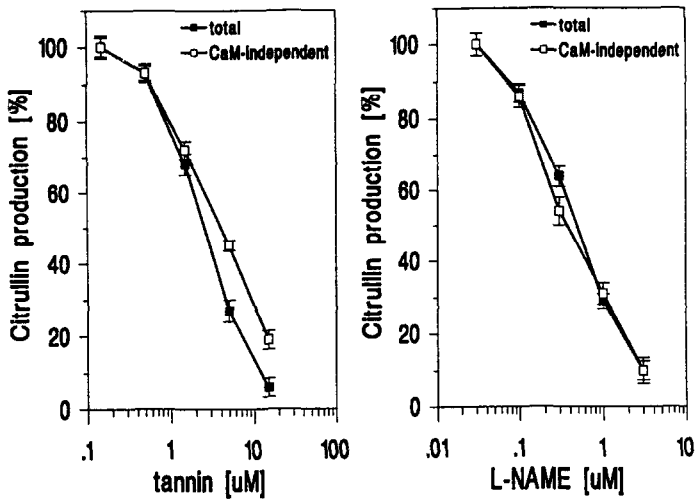

Fig. 6. The effect of calmodulin-antagonists on the L-NAME and tannin inhibition of eNOS. eNOS activity was measured in the presence of various concentrations of either L-NAME or tannin. The experiments were carried out in the absence (total) or the presence (CaM-independent) of 200 μM of the calmodulin antagonist CGS 9443. The data are means ± SEM, N = 4; control values for total activity (in the absence of CGS 9343) and calmodulin-independent activity (in the presence of CGS 9343) were set to 100%. Total activity was 3530 ± 35 cpm of L-citrulline produced in 10 min. CaM-independent activity was 1447 ± 26 cpm/10 min.

this observation. The calmodulin antagonist CGS 9443 was found to inhibit completely the NOS activity of the neuronal constitutive isoform with an IC_{50} of approx. 10 μM and, as expected, had no effect on iNOS activity (see Fig. 5). eNOS activity could also be inhibited by addition of CGS 9343 (see Fig. 5), but a portion of this activity was resistant to the action of the calmodulin antagonist (up to 300 μM). The portion of eNOS activity resistant to the calmodulin antagonist was still fully dependent on Ca^{2+} (not shown). Figure 6 shows that L-NAME inhibited residual (calmodulin-independent) activity and total eNOS activity with an identical potency. Interestingly, in the presence of the calmodulin antagonist, residual enzyme activity became a little more resistant to tannin inhibition (see Fig. 6).

DISCUSSION

Among the natural flavonoids tested in this study, only tannin and quercetin showed a direct inhibition of the activity of eNOS. The effect was concentration dependent and completely reversed by dilution. Tannin, the more potent of the two compounds, inhibited NOS activity in the low micromolar range and showed some selectivity towards the endothelial isoform (20–30-fold higher concentrations were needed to inhibit nNOS and iNOS). One should mention that while nNOS and iNOS are essentially found in the soluble cytosolic fraction, most of the eNOS activity is recovered in the particulate fraction from endothelial cell homogenates. Thus, in this study different cellular fractions (i.e. soluble versus particulate fractions) were utilized as a source of enzyme and this might lead to an overinterpretation of the selectivity of tannin inhibition. However, very similar IC_{50} values for tannin inhibition were obtained when utilizing either the soluble or the particulate portions of eNOS (not shown). Thus it is likely that the difference in potency is rather due to intrinsic differences in the three isoforms than to different preparation conditions. Several experiments were carried out to clarify the mechanism of the inhibitory action exerted by tannin. NOS catalyses a very complex reaction with the interplay of several co-substrates and co-factors so that inhibitors could have various possible modes of action. The most common class of inhibitors interferes with the binding and utilization of the substrate L-arginine. A kinetic analysis of the action of tannin on the enzyme showed no effect on L-arginine binding. Tannin inhibition was also unaffected over a wide range of NADPH and tetrahydrobiopterin concentrations, indicating that neither did tannin compete with the action of these co-substrates and co-factors. Another possible way of inhibiting NOS activity is by interfering with the regulation by the Ca^{2+}–calmodulin complex, another essential co-factor. Since the activity of iNOS does not depend on the presence of Ca^{2+} ions, it was originally supposed that Ca^{2+}–calmodulin binding was not required for its activity. Later on, however, it was shown that calmodulin is permanently bound to iNOS even at the lowest cytosolic concentrations of Ca^{2+} [13]. The strong and irreversible interaction of calmodulin is likely to be necessary for the enzymatic activity of iNOS as well. Recently, information on the possible mode of action of calmodulin on NOS has become available. The binding of calmodulin to nNOS (and possibly also to other isoforms) has been shown to facilitate the transfer of NADPH-derived electrons to the heme group, thus stimulating NO synthesis

[14]. Two major interpretations could be envisaged to explain this interesting finding. First, calmodulin, by acting via a completely unusual mode of action, could be directly involved in the flow of electrons. Alternatively, it is quite possible that the binding of calmodulin to NOS induces a rearrangement of functional domains so as to approach the NADPH and heme binding regions and enable the flow of electrons. This second mode of action would be reminescent of that described for a variety of other calmodulin-regulated enzymes, whereby calmodulin binding removes an auto-inhibitory domain from the active site [15, 16]. We postulate that the inhibition of NOS by tannin is partly due to interference with the activation by calmodulin. It is unlikely that tannin could displace calmodulin from its binding domain on the enzyme (on iNOS in particular), but it could prevent the change in conformation that normally follows the interaction of calmodulin with the various NOS. Crude particulate eNOS preparations display the peculiar characteristic of being partially resistant to the action of calmodulin antagonists (see Fig. 5 and Ref. 11). Based on this observation, Forstermann *et al.* [11] postulated that there may be some differences in the binding or calmodulin requirement between endothelial and brain NOS isoforms. However, after solubilization and purification, eNOS, similar to the soluble nNOS isoform [8], becomes completely calmodulin dependent and fully inhibitable by calmodulin antagonists [7, 17]. Thus it is possible, that another hitherto unknown stimulatory mechanism besides calmodulin (such as interaction with lipids or a protein or a particular phosphorylation state) is effective in the crude preparations of eNOS. This would somehow compensate for the loss of activity following calmodulin removal and partially maintain activity. (It is noteworthy that addition of exogenous lipids was found to stimulate eNOS [18].) The fact that tannin more effectively inhibited the calmodulin-dependent portion of the activity of eNOS (see Figs 4 and 6) indicates that the inhibitor preferentially interferes with the activation associated with the conformational changes occurring upon calmodulin binding.

A stimulation (rather than an inhibition) of eNOS activity could have been anticipated, based on the vasodilatatory effect of tannin and quercetin at the tissue level [4, 6], It may be of interest in this context that in another study quercetin has been described as an inhibitor of endothelial-mediated vasodilation which would be consistent with our results [19]. Tannin is not a single chemical entity and commercially available tannin preparations do not specify the various contents in hydrolysable and condensed tannins nor give the source of biological material. It is possible, therefore, that the variability of tannin preparations could explain the apparent contradiction between the inhibitory effect of tannin on NO production reported in the present communication and the stimulatory effect postulated by others [4, 6]. Another important point to consider is that previous work [4, 6] described the effect of tannin on intact cells or tissues whereas our study dealt with subcellular fractions and was devoid of cell permeation problems. Finally, one should consider that plant phenols display a multiplicity of other biochemical actions, such as inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase in skeletal muscle [20, 21], and stimulation of cardiac sarcoplasmic reticulum Ca^{2+}-ATPase [21], and inhibition of the Na,K-ATPase [22], and of phosphodiesterase [23], which complicate the interpretation of the effects at the cell or tissue levels.

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