academicJournals

Vol. 5(7), pp. 132-141, July 2013
DOI: 10.5897/JPP2013.0272
ISSN 2141-2502 ©2013 Academic Journals
http://www.academicjournals.org/JPP

Journal of Pharmacognosy and Phytotherapy

Full Length Research Paper

Nitric oxide-dependent vasodilation and intracellular Ca^{2+} concentration increase induced by 6,8-dihydroxy-4'-methoxyflavone in rat aorta

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Accepted 10 June, 2013

In this study, we attempted to evaluate the role of endothelium-derived hyperpolarizing factor (EDHF) in the vasorelaxant response induced by 6,8-Dihydroxy-4'-methoxyflavone (DMF) in isolated rat aorta rings by using functional and biochemical approaches. Thoracic aortic rings were isolated and suspended in organ baths, and the effects of DMF were studied by means of isometric tension recording experiments. Nitric oxide (NO) was detected by ozone-induced chemiluminescence. The technique used to evaluate changes in intracellular ($[Ca^{2+}]_i$) in intact endothelium was determined by open aortic ring and loaded with 16 µmol Fura-2/AM for 60 min at room temperature, washed and fixed by small pins with the luminal face up. *In situ* electrical conductivities (ECs) were visualized by an upright epifluorescence Axiolab microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Zeiss × 63 Achroplan objective (water immersion, 2.0 mm working distance, 0.9 numerical apertures). ECs were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. In aortic rings with intact endothelium pre-contracted with norepinephrine (NE) (10^{-4} M), the addition of DMF (10^{-8} to 10^{-4} M) induced vasorelaxation in a concentration-dependent manner; in endothelium-denuded rings, the relaxant response induced by DMF was almost completely abolished suggesting that vasorelaxation was endothelium-dependent. DMF (10^{-4} M) was able to significantly increase NOx levels. This effect was completely abolished after removal of the vascular endothelium. DMF (100 µM) caused a slow, long-lasting increase in $[Ca^{2+}]_i$. These results further support the hypothesis that DMF can induce activation of the NO/sGC/cGMP pathway, as suggested by functional studies. The results of the present study, using combined functional and biochemical *in vitro* approaches, indicated that DMF relaxes pre-contracted isolated rat aortic rings. Such a vasorelaxation was an endothelium-dependent effect, via the NO/sGC/cGMP pathway. This result also suggests that DMF causes a slow influx of extracellular Ca^{2+}. Release from the intracellular Ca^{2+} stores and an inhibition of Ca^{2+} extruding mechanisms.

Key words: 6,8-dihydroxy-4'-méthoxyflavone, aorta, nitric oxide (NO), Ca^{2+} signalling, endothelium, NO–cGMP pathway, vasodilation.

INTRODUCTION

Some isolated compounds have been reported to mediate a plethora of relevant biological activities,

especially those related to vascular tone control. The bark parts of several species of Rutaceae, mainly of the genus Vepris, are considered aromatic and medicinal (Ngassoum et al., 2007; Hamawa et al., 2010). Phytochemical investigation of the stem barks of *Vepris heterophylla* (Engl.) R. Let. (Rutaceae), a medicinal plant used empirically in Cameroon by traditional healers in the treatment of various illnesses such as cardio-vascular disorders, especially arterial hypertension, and renal disorders, led to the isolation of the flavonoid, 6,8-dihydroxy-4'-methoxyflavone (DMF). To our knowledge, there have been no reports on the biological activity of this molecule. Flavonoids exhibit diverse biological effects, including inhibition of protein kinase C, inhibition of cyclic nucleotide phosphodiesterase, decrease in Ca^{2+} uptake, and vasodilators actions. For the purpose of using 6,8-dihydroxy-4'-methoxyflavone, which remains as an unused resource after extraction from the bark of the trunk of *V. heterophylla* (Rutaceae) plant use as a medicinal therapeutic to treat hypertension and renal disorders (Toshiaki et al., 2006).

Endothelial cells play an important role in the control of vascular tone through the expression of constitutive or induced molecules on their surfaces, and the synthesis and secretion of soluble mediators are responsible for vascular homeostasis (Widlansky et al., 2003). Endothelial cells respond to a variety of neurohumoral and physical stimuli to release endothelium-dependent vasodilators such as endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980; Furchgott and Vanhoutte, 1989), prostacyclin (PGI2) (Jaffe, 1985; Vanhoutte et al., 1986) and endothelium-derived hyperpolarizing factor (EDHF) (Beny and Brunet, 1988; Feletou and Vanhoutte, 1988; Taylor and Weston, 1988; Nakane et al., 1991).

It is generally accepted that the release of EDRF, PGI2 and EDHF may have important physiological roles as dilator mediators in certain vessels. Other important mediators for relaxing the vascular smooth muscle are cyclic nucleotides (Murad, 1986). It is recognized that nitrovasodilators exert their effects on vascular smooth muscle by activating guanylate cyclase and increasing cyclic guanosine monophosphate (GMP), and that P-adrenoceptor agonists act by stimulating adenylate cyclase and increasing cyclic adenosine monophosphate (AMP). NO is derived from the oxidation of L-arginine by Ca^{2+}-calmodulin–dependent nitric oxide synthase (NOS), an enzyme constitutively expressed in the endothelial cells (Bredt et al., 1992; Yi-Ching et al., 2005). Furthermore, NOS can be activated by an Akt/PKB, Ca^{2+}-independent pathway (Arnold et al., 1997; Lowenstein et In this study, we attempted to evaluate the role of endothelium-derived factors in the vasorelaxant response induced by DMF in isolated rat aorta rings by using functional and biochemical approaches.

MATERIALS AND METHODS

Animals

Wistar rats (250 to 350 g) were used for all experiments. Animals were housed under conditions of controlled temperature (20 to 24°C) and humidity (55 ± 10%). In addition, they had free access to food (Harlan Teklad, Global diets, Pavia, Italia) and tap water *ad libitum*.

The animal handling was under the control of the veterinary surgeon of the University of Pavia. Experimental protocols and procedures were approved by the institutional Animals Care and Use Committee and the research was approved by the Ethical Committee of the University of Pavia.

Drug administration

The following drugs were used: ATP, 6,8-dihydroxy-4'-methoxyflavone, Lantan 3+, dimethylsulfoxide (DMSO), norepinephrine, acetylcholine chloride, atropine sulfate, Nω-nitro-L-arginine methyl esther (L-NAME), indomethacin, L-arginine, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), all from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions were prepared in distilled water and kept at -4°C. DMF was solubilised in distilled water and diluted to the desired concentrations with distilled water just before use. ODQ was dissolved in DMSO. The other compounds were dissolved in distilled water. The final concentration of DMSO in the bath never exceeded 0.1%, and has no effect when tested in control preparations (data not shown).

Chemical solutions

The composition of the Tyrode's solution used was 158.3 mM NaCl, 4.0 mM KCl, 2.0 mM $CaCl_2$, 1.05 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 10.0 mM $NaHCO_3$, and 5.6 mM glucose. The physiological salt solution (PSS) had the following composition (in mM) 150 NaCl, 6 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Hepes, pH 7.4. In Ca^{2+}-free solution, Ca^{2+} was substituted with 2 mM NaCl, and 0.5 mM EGTA was added. Both solutions were titrated to pH 7.4 with NaOH. Fura-2/AM was obtained from Molecular Probes (Molecular Probes Europe BV, Leiden, The Netherlands). All other chemicals were purchased from Sigma. Medium exchange and administration of agonists or other drugs were performed by removing the bathing medium (2 ml) and adding the desired solution. The medium could be exchanged quickly without producing artefacts in the fluorescence signal because a small meniscus of liquid remained between the tip of the objective and the facing surface of the cover slip.

Plant collection and preparation of the extract

The stem bark was collected in February, 2010 in the Mokolo (Far North Region) in Cameroon (10° 39.214' N, 14° 24.145' E, 375 m of traditional uses. A sample was identified at the National Herbarium Cameroon (NHC) where a voucher specimen is deposited. The stem bark was cut air-dried and crushed. 2.5 kg of powder of the plant material was introduced into the extraction solvent at room temperature. The extraction lasted for 48 h. After decantation and filtration, the macerate was collected in a altitude). This region has annual average humidity of 73% and an average temperature of 29°C. This plant was selected on the basis volumetric flask. The

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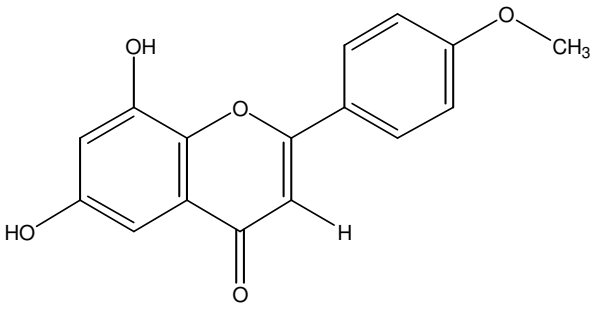

Figure 1. 6,8-Dihydroxy-4'-methoxyflavone structure.

operation was repeated two times with the solvent. Each extract was concentrated to dryness under reduced pressure using a rotary evaporator (BÜCHI). From this procedure, 175 g of ethyl acetate extract were obtained and kept at -0.5°C until use.

Preparation of fractions and isolation of the molecule

Fifty grams of the organic extract were subjected to flash chromatographic fractionation on a silica gel column (1.5 m length) of 40 ml eluted with hexane followed by gradient mixtures of hexane-ethyl acetate-methanol. Fractions of approximately 150 ml were collected and grouped on the basis of thin layered chromatography (TLC) on aluminum plate. Purification of the crystals obtained in the series of fractions was done by washing a solvent mixture of EtOAc-Hex (25/75) and preparative TLC, the compound 6,8-dihydroxy-4 '-methoxyflavone was obtained.

Spectral characterization of DMF

The use of spectral techniques: Nuclear magnetic resonance (NMR) to one or two dimensions, mass spectrometry (MS), UV-Visible spectroscopy and high performance liquid chromatography (HPLC) allowed us to clarify and determine the structure of the secondary metabolite isolated from the extract with ethyl acetate bark of the trunk of *V. heterophylla* (Rutaceae). This compound crystallizes as yellow flakes in the system Hex/AcOEt (30/70). Its UV spectrum shows respectively the wavelength λ = 248.7 nm (band II, cinnamoyl cycle), λ = 300 nm (band I, benzoyl ring) and the absorbance A = 2.625, A = 2.445 consider leaving structure flavonoid type. Its formula $C_{16}H_{12}O_5$ with 11 instaurations was deduced from its mass spectrum (TOF MS ES^+ 2.26 e4) in a positive ionization mode that shows pseudomolecule ions at m/z 286.14 $[M+H]^+$, 593.22 $[2M+Na]^+$; 878.29 $[3M+Na]^+$; 1163.49 $[4M+Na]^+$ involving its molecular weight 284 u. The ^{13}C NMR spectrum shows a mass of 18 carbon signals including a carbonyl at 180.77 ppm, eleven aromatic carbons in the range 86.68 to 159.30 ppm; a signal at 55.99 ppm characteristic of the methoxy group. The ^1H NMR spectrum shows five protons of aromatic type including 1 to 6.23 ppm is typical of H-2 protons of flavones, a signal to 3.72 ppm characteristic of the methoxy group; a signal at 8.45 ppm characteristic of the hydroxyl groups in position 6 and 8. The analysis of 2D COSY spectrum shows correlations between the signal at 7.85 and 7.23, 7.23 and 7.23, 7.85 and 7.85 ppm. The heteronuclear multiple-bond correlation spectroscopy (HMBC) spectrum allowed us to highlight the correlations between 7.85 and 126.57 ppm and 141.96, 115.57 and 7.23 ppm. The heteronuclear single quantum coherence (HSQC) spectrum shows the correlations 7.85 and 130 ppm, 7.23 and 114.57 ppm, 7.23 and 121 ppm. Spectroscopic data described in the literature are consistent with the values of the chemical shifts of the compound 6,8-dihydroxy-4 '-methoxyflavone (Gomes et al., 1983) (Figure 1).

Preparation of isolated rat superior aorta rings

Wistar rats were sacrificed by stunning and bleed. The thoracic and abdominal aorta were dissected out and perfused with Physiological Salt Solution (PSS). The superior aortic artery was removed and cleaned from connective tissue and fat. Rings (0.5 mm) were obtained and placed in Fura-2/AM for 1 hour; the rings were removed and stored in PSS for 30 min, at room temperature (22 to 24°C). When appropriate, the endothelium was removed by gently rubbing the intimal surface of the vessels. Rings (1 to 2 mm) were obtained and placed in physiological or Tyrode's solution, maintained to 37°C, and gassed with carbogenic mixture (95% O_2 and 5% CO_2) and maintained at pH 7.4. The preparations were stabilized under a resting tension of 1 g for 1 h; during this time, the solution was changed each 15 min to prevent the accumulation of metabolites (Flesch et al., 1998). The force of isometric contractions was recorded by a force transducer (Miobath-4; WPI, Sarasota, FL, USA) coupled to an amplifier-recorder (Miobath-4, WPI) and to a personal computer equipped with an analogue to digital converter board. The presence of functional endothelium was assessed by the ability of acetylcholine (10 µM) to induce more than 90% relaxation of vessels pre-contracted with norepinephrine (10 µM) and the absence, less than 10%, of relaxation to acetylcholine was taken as an evidence that the vessel segments were functionally denuded of endothelium (Dimo et al., 2005; Ntchapda et al., 2009). Vessels with intact functional endothelium and endothelium-denuded vessels showed no significant difference in the magnitude of contraction. The preparations were exposed to L-NAME (100 µM), a nitric oxide synthase (NOS) inhibitor (Moncada et al., 1993); L-NAME (100 µM) plus L-arginine (1 mM), the endogenous substrate of NOS (Toda et al., 2003); ODQ (10 µM), a soluble guanylyl cyclase (sGC) inhibitor (Garthwaite et al., 1995); or indomethacin (10 µM), a cyclo-oxygenase (COX) inhibitor (Moncada et al., 1991), plus atropine (1 nM), a non-selective muscarinic-receptor antagonist (Shiraki et al., 2001), used separately. These inhibitors were added 30 min before the application of norepinephrine. In the tonic phase of the second contraction, DMF (10^{-7} to 10^{-4} M) was cumulatively added to the preparations. Inhibition was calculated by comparing the response of DMF before and after the addition of the inhibitor or antagonist.

Determination of NO level

The rat aorta was removed as described above, and rings 1 to 2 mm in width were placed in a 12-hole plate containing Tyrode's solution (0.7 ml/hole) and placed at 37°C for 40 min. Following incubation with the drugs, the middle of each well was collected and used for the determination of Nox and the rings weighed. The total amount of NOx in the medium was determined using the purge system of Sievers instruments (model NOA 280i, Boulder, CO, USA) (Braman et al., 1989). A saturated solution of vanadium chloride (VCl 3) in 1 M HCl was added to the nitrogen-bubbled purge vessel fitted with a cold water condenser and a water jacket to allow heating of the reagent to 90°C using a circulating bath. HCl vapors were removed by a gas bubbler containing sodium hydroxide (1 M). Flow of gas into the detector was controlled by a needle valve adjusted to yield a constant pressure. When the detector signal is stabilized, samples were injected into the purge ring to react with the reagent, thereby converting Nox to NO, which was then detected by ozone-induced chemiluminescence. NOx concentrations were calculated by comparison with a standard solution of sodium nitrate. Values for the control of the baseline were obtained from the aorta ring before the administration of

drugs. Rings (with or without the vascular endothelium) were then incubated with norepinephrine (10 µM) for 20 min. After this period, in each hole, 10 µM of acetylcholine was administered as a positive control and the DMF as negative control. After half an hour, the center of each hole was recovered and used for the determination of NOx, as described. To investigate whether NO production was Ca^{2+}-dependent, DMF was applied in an extracellular Ca^{2+}-free solution and in an extracellular Ca^{2+}-free solution after depletion of intracellular Ca^{2+}-store by:

1. Applying La^{3+} (100 µM) in Ca^{2+}-free solution for 30 min, and then washing the ring by extracellular Ca^{2+}-free solution and applying DMF in extracellular Ca^{2+}-free solution.
2. Applying adenosine triphosphate (ATP) 300 µM in Ca^{2+}-free solution for 10 min, washing and incubating in Ca^{2+}-free solution for 20 min and then washing the ring by extracellular Ca^{2+}-free solution.

Ca^{2+}-free solution can be obtained by both avoiding Ca^{2+} addition and adding EGTA [final concentration 0.5 mM (pH 7.4)]

Intracellular [Ca^{2+}] evaluation

The technique used to evaluate changes in intracellular ($[Ca^{2+}]_i$) in intact endothelium has previously been described (Moccia et al., 2002; Yuly et al., 2010). The aortic ring was opened and loaded with 16 µmol Fura-2/AM for 60 min at room temperature, washed and fixed by small pins with the luminal face up. *In situ* ECs were visualized by an upright epifluorescence Axiolab microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Zeiss × 63 Achroplan objective (water immersion, 2.0 mm working distance, 0.9 numerical apertures). ECs were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. The exciting filters were mounted on a filter wheel (Lambda 10, Sutter Instrument, Novato, Calif., USA). Custom software, working in the LINUX environment, was used to drive the camera (Extended-ISIS Camera, Photonic Science, Millham, UK) and the filter wheel, and to measure and plot online the fluorescence from 10 to 15 rectangular 'regions of interest' (ROI) enclosing one single cell. $[Ca^{2+}]_i$ was monitored by measuring, for each region of interest, the ratio of the mean fluorescence emitted at 510 nm when exciting alternatively at 340 and 380 nm (shortly termed 'ratio'). An increase in $[Ca^{2+}]_i$ caused an increase in the ratio. The experiments were performed at room temperature (21 to 23°C).

Data analysis

Mean values are presented together with standard error of the mean and the whole number of tested cells 'n' or number of experiments. Statistical significance ($p < 0.05$) was evaluated by the Student t-test and one-way analysis of variance (ANOVA), using Origin graph, (Microcal Origin 6.0) software version 6.0. Tracings shown in the figures are single cell recording. The potent vasorelaxation (pD2) value was calculated by non-linear regression. Emax is the maximal relaxation or maximal increase in $[Ca^{2+}]_i$ at the highest concentration used. The slope with correlation coefficient was measure by the Fit Linear regression. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Relaxant activity of DMF

The mechanisms of DMF in causing vasorelaxation, and $[Ca^{2+}]_i$ increase in rat isolated thoracic aorta was investigated. In aortic rings with intact endothelium pre-contracted with norepinephrine (10^{-4} M), the addition of DMF (10^{-8} to 10^{-4} M) induced vasorelaxation [pD2 = 10.43 ± 3.29] in a concentration-dependent manner in endothelium-denuded rings, the relaxant response induced by DMF was almost completely abolished (pD2 = 1.08 ± 0.34) suggesting that vasorelaxation was endothelium-dependent (Figure 2). The maximal relaxant effects (Emax) of DMF in the presence and absence of the endothelium are reported in Table 1. In the presence of the NOS inhibitor L-NAME (100 µM), the relaxation induced by DMF in rings with or without endothelium was similar to that induced in endothelium-denuded rings (Figure 3). The inhibitory effect of L-NAME (100 µM) was completely reversed by the addition of the biological substrate of NO synthase L-arginine (1 mM) (pD2 = 09.93 ± 0.22) (Figure 3). In the presence of ODQ (10 µM), an inhibitor of the soluble guanylyl cyclase (sCG**)**, the relaxation induced by DMF (10^{-8} to 10^{-4} M) was almost completely abolished (pD2 = 1.01 ± 0.32) (Figure 4 and Table 1). We also investigated the contribution of relaxant arachidonic acid derivatives and activation of the muscarinic receptors. In rings pre-incubated with indomethacin (1 µM) plus atropine (1 nM), the relaxation induced by DMF was not significantly different from the relaxation induced in endothelium-intact rings (pD2 = 11.16 ± 0.36) (Figure 4 and Table 1).

NO production

In isolated rat aorta rings, DMF (10^{-4}M) was able to significantly increase NOx levels. This effect was completely abolished after removal of the vascular endothelium (Figure 5). Interestingly, when submitted to the same experimental conditions, acetylcholine (10^{-5} M), used as positive control for NO production, induced an increase in NO- levels about 50% of that induced by 10^{-4} M DMF

Ca^{2+} signals

Ca^{2+} signalling induced by DMF in *in situ* endothelium of aortic rings was also evaluated. DMF (100 µM) caused a slow, long-lasting increase in $[Ca^{2+}]_i$ (Figure 6). The amplitude of the Ca^{2+} signal evoked by a high ATP concentration, known to be able to induce NO synthesis, was comparable with the increase in $[Ca^{2+}]_i$ evoked by DMF (Figure 7), suggesting that also DMF could be able to evoke a Ca^{2+}-dependent NO synthesis. In Ca^{2+}-free extracellular solution, the slow increase in $[Ca^{2+}]_i$ was still present, but with a slope (1.17, correlation coefficient = 0.95; n = 8) (Figure 8) much smaller than control values (8.31, correlation coefficient = 0.92; n=9), suggesting that Ca^{2+} influx is involved. The introduction in the incubation medium of DMF 20 min before the higher magnitude response produced by ATP and 100 s before the addition

Table 1. Relaxation induced by DMF in the different experimental conditions.

Experimental condition	Emax (% relaxation)	n
-Intact endothelium (E+)	84.22±6.11	6
-E+ plus L-NAME (100 µM) plus L-arginine (1000 µM)	70.02±1.49	6
-E+ plus L-NAME (100 µM)	12.31±1.84***	6
-E+ plus ODQ (10 µM)	11.21±1.50***	6
-Endothelium denuded	18.49±2.52***	6
-Endothelium denuded plus L-NAME (100 µM)	16.75±1.94***	6
-E+ indomethacin (1µM) plus atropine (1 nM)	79.98±4.53	6

n, number of experiments. *** < 0.001 versus control.

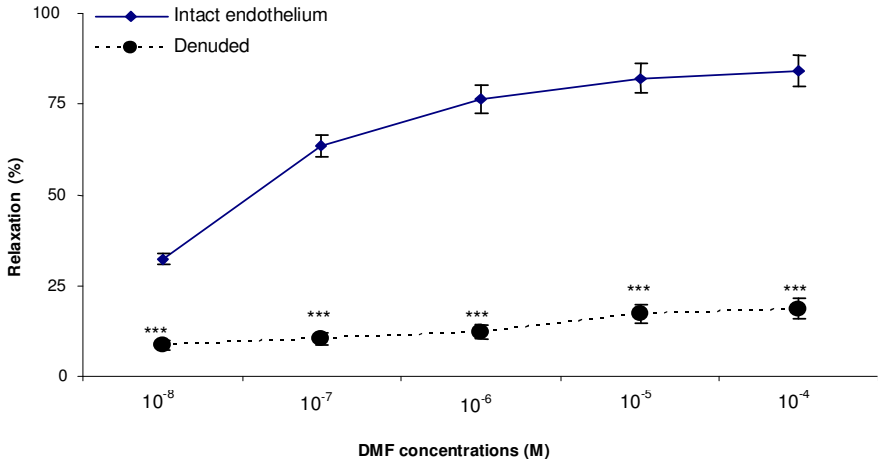

Figure 2. Relaxation induced by DMF in endothelium-intact (n = 6) or endothelium-denuded (n = 6) rat aortic rings. Values are mean ± SEM., *** P < 0.001.

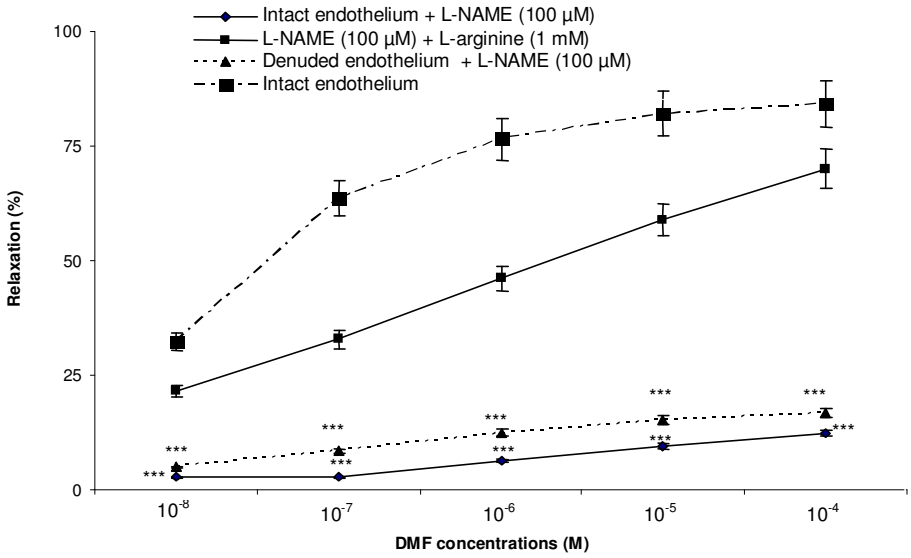

Figure 3. Relaxation induced by DMF (10^{-8} to 10^{-4} M) in endothelium-intact rings (n = 6), endothelium-denuded rings, after pre-treatment with L-NAME (100 µM; n=6), and endothelium-intact rings after pre-treatment with L-NAME (100 µM) plus L-arginine (1 mM) (n = 6). Values are mean ± S.E.M., *** P < 0.001 versus endothelium-intact ring + L-NAME.

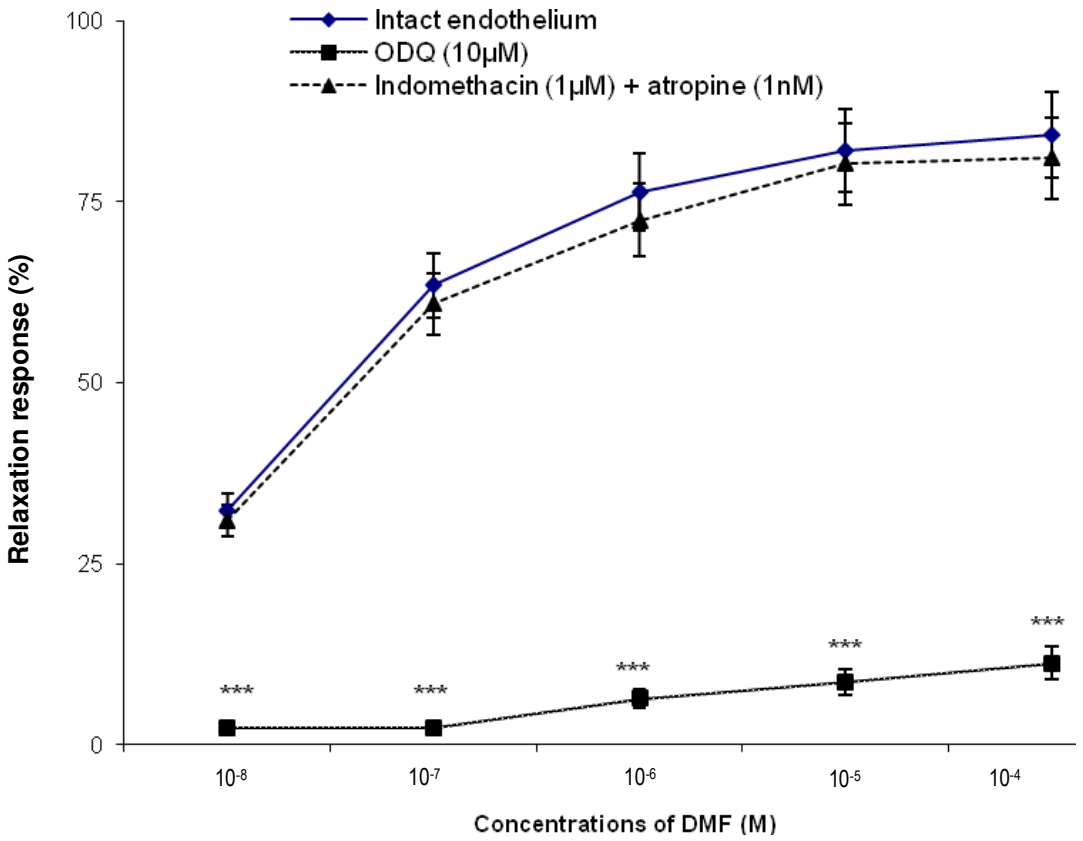

Figure 4. Relaxation induced by DMF (10^{-8} to 10^{-4}M) in endothelium-intact (n = 6) aortic rings, after (n = 6) pre-treatment with ODQ (10 µM), and after (n = 6) pre-treatment with indomethacin (1 µM) plus atropine (1 nM). Values are mean ± SEM. *** P < 0.001 versus endothelium-intact ring.

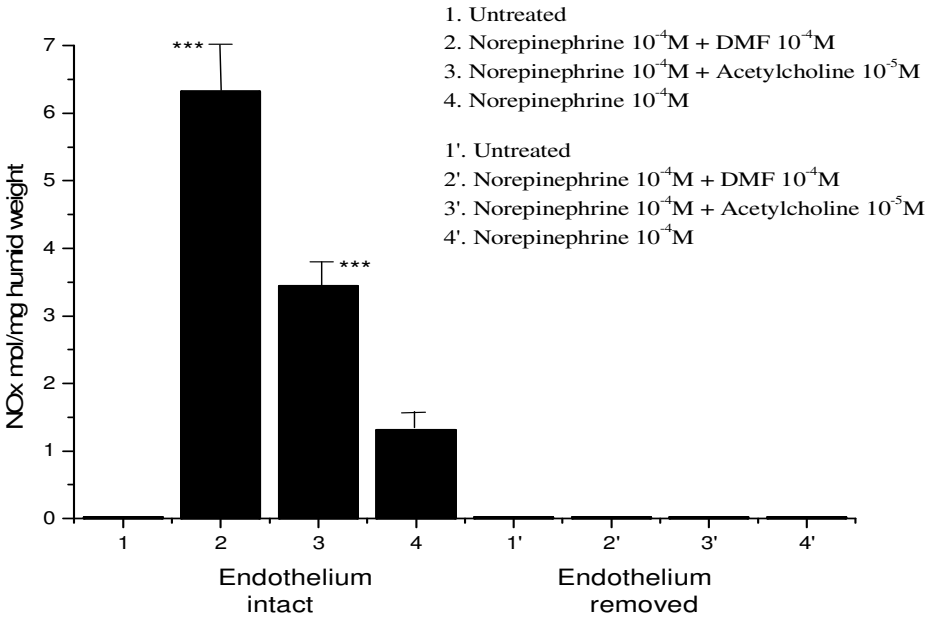

Figure 5. Effects of DMF (10^{-4} M) and acetylcholine (ACh, 10^{-5} M) on NOx level in rat aorta rings pre-contracted with norepinephrine (10^{-4} M), in aortic rings with intact endothelium (n = 5) or removed endothelium (n = 5). Mean ± SEM. *** P < 0.001 versus norepinephrine,

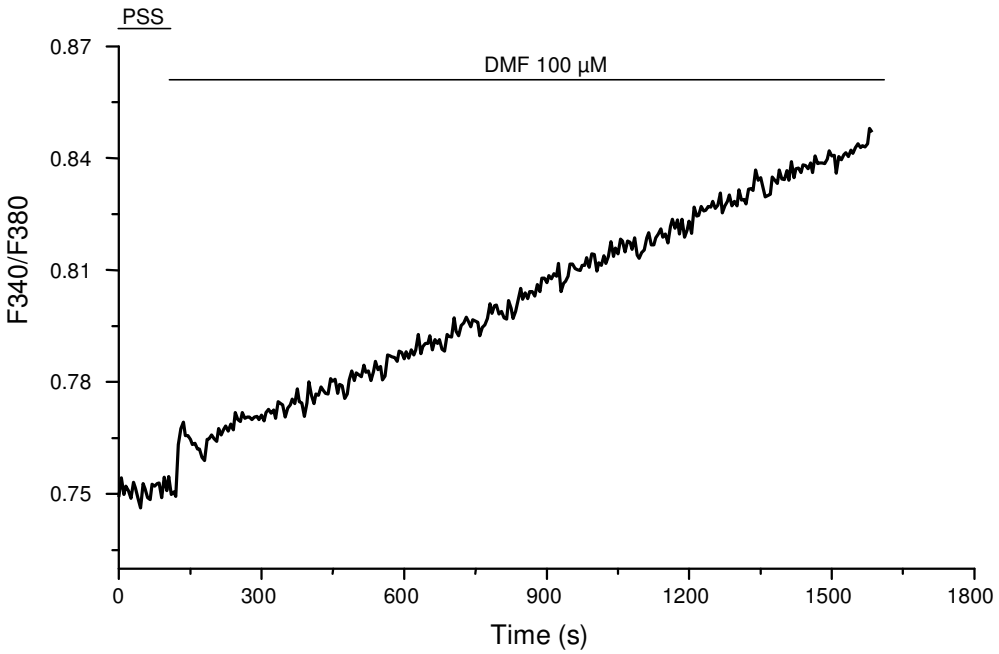

Figure 6. Effect of DMF (100 µM) on the intracellular Ca^{2+} concentration of aorting ring endothelial cells (single cell tracing)

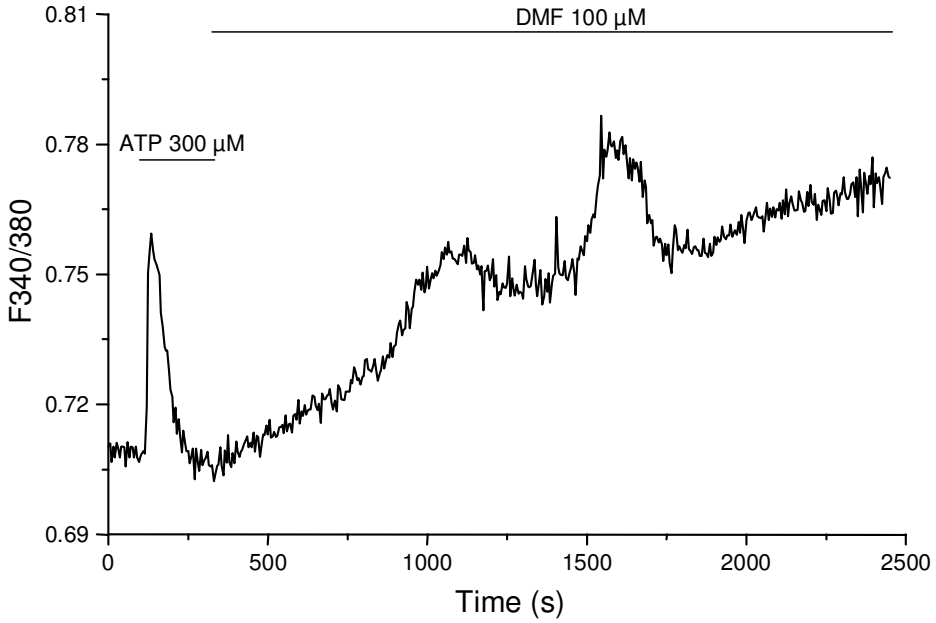

Figure 7. Effect of ATP (300 µM) and DMF (100 µM) on the intracellular Ca^{2+} concentration of aorting ring endothelial cells (single cell tracing).

of DMF did not provoke any significant modifications of the amplitude effects of DMF (data no shown). In the presence of La^{3+}, the higher slope due to DMF (7.93, correlation coefficient = 0.97; n = 8) was completely abolished as shown in Figure 9, suggesting that DMF-induced intracellular influx Ca^{2+} involves the participation of channels calcium. These results further support the hypothesis that DMF can induce activation of the NO/sGC/cGMP pathway, as suggested by functional studies.

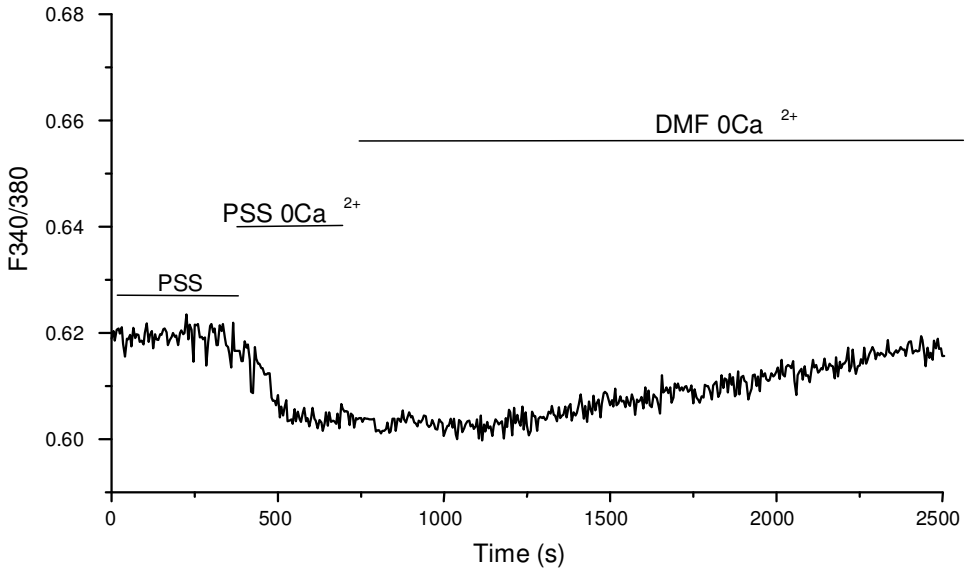

Figure 8. Effect of DMF (100 µM) on the intracellular Ca^{2+} concentration of aorting ring endothelial cells in the presence (PSS) and absence of extracellular Ca^{2+} ($0Ca^{2+}$) (single cell tracing).

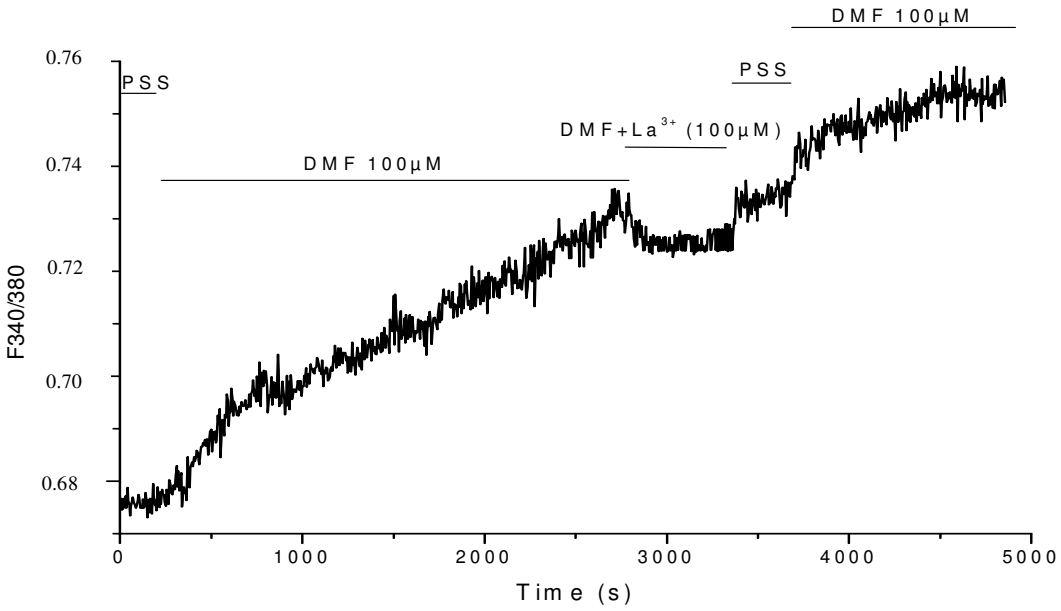

Figure 9. Effect of DMF (100 µM) on responses to the influx of extracellular Ca^{2+} after (n = 8) pre-treatment of the rings with La^{3+} (100 µM).

DISCUSSION

In this paper, we have shown that DMF, a flavonoid isolated from stem bark of *V. heterophylla* induces a strong, dose-dependent relaxation of the rat aortic rings pre-contracted with norepinephrine. Furthermore, DMF relaxing activity is fully dependent on endothelial cells via the NO-cGMP pathway. Indeed, mechanical removal of endothelium abolished the relaxant response induced by DMF, suggesting that DMF activates an endothelium-dependent mechanism (Table 1 and Figure 2). To elucidate a mechanism, we first evidenced that inhibition of NO synthesis by the L-arginine analogue L-NAME abolished the vasorelaxation induced by DMF. Furthermore, in rings pre-incubated with L-NAME plus L-arginine, the vasorelaxante response by DMF was

completely reversed (Table 1 and Figure 3), suggesting that the DMF effect is mediated by a mechanism involving endothelium-derived NO. The action of NO, as a vasodilator, is mediated by the activation of vascular smooth muscle sGC, an enzyme that forms the second messenger cGMP, which activates a cGMP-dependent protein kinase (PKG) (Arnold et al., 1997; Lowenstein et al., 1994). To determine if DMF-induced relaxation involves the participation of a cGMP pathway, the preparations were pre-treated with ODQ, a soluble guanylyl cyclase inhibitor (Garthwaite, 1995). In these conditions, the vasorelaxation induced by DMF was almost completely abolished (Figure 4).

We also excluded that the endothelium-dependent vasorelaxant response may also involve the release of COX-derived products, such as PGI2, via the cyclo-oxygenase pathway (Furchgott and Zawadzki, 1980; Rapoport, 1986; Archer, 1994) or muscarinic receptor activation. To investigate the participation of theses pathways, we pre-treated the vessels with indomethacin (10 µM) plus atropine (1 nM). In this condition, DMF's effects were similar to that obtained in control conditions, suggesting that muscarinic-receptor activation or COX-derived products are not involved (Figure 4).

Taken together, these data provide strong evidence that DMF induces potent endothelium-dependent relaxations involving NO release. To further confirm such a hypothesis, a biochemical assay using a very sensitive technique for NOx analysis was performed (Chen, 1988; Braman et al., 1989). Since the half-life of NO is very short (a few seconds), nitrate, nitrite, and other nitroso compounds (NOxs), which are stable metabolites of NO, are frequently measured to determine NO production (López-Ramos et al., 2005). In endothelium-intact aorta rings pre-contracted with norepinephrine, DMF (10^{-4} M) and acetylcholine (10^{-5} M, used as positive control) significantly increased NOx levels. As illustrated in Figure 5, in response to 10^{-4} M DMF, NOx levels increased to virtually the same level as that reached after 10^{-5} M acetylcholine. Inversely, in rings in which the vascular endothelium was removed, no effect of DMF on NOx levels was observed, excluding a possible NO-donating effect induced by the compound.

It is well known that activation of constitutive endothelial NOS may be Ca^{2+} dependent. DMF caused a slow, long lasting increase in the $[Ca^{2+}]_i$ of aortic endothelial cells. Such a slow $[Ca^{2+}]_i$ increase was very limited in Ca^{2+}-free extracellular medium (the slope in Ca^{2+}-free extracellular medium was only 14% of the slope in normal solution). This result suggests DMF causes a slow influx of extracellular Ca^{2+} release from the intracellular Ca^{2+} stores and an inhibition of Ca^{2+} extruding mechanisms. Ca^{2+} is known as an important regulatory element for many cellular processes; it acts either directly as a 2nd messenger or for the maximum activation of other enzymes in the signal cascade (Putney 1993; Berridge, 1997).

DMF (10^{-4} M), administrated in Ca^{2+} free medium did not increase intracellular influx Ca^{2+}. What suggest that DMF would act on the calcium channels by stimulating their openings, thus allowing a massive entry of Ca^{2+} into the cell. To determine if DMF-induced intracellular influx Ca^{2+} involves the participation of channels calcium, the preparations were pre-treated with La^{3+} the non-specific calcium channel antagonists, which usually blocks calcium influx and calcium-related metabolic functions such as trans-membrane Ca^{2+} transport within excitable tissues (Fitzpatrick, 1990), inhibited higher amplitude due to DMF-induced Ca^{2+} release as illustrated in Figure 9. Negative effects of lanthanum on cells are attributed to its blockage of Ca^{2+}-channel on the cell membrane, and on this basis, a higher concentration of lanthanum (100 µM) has been extensively used to inhibit Ca^{2+} influx to investigate various Ca^{2+}-dependent cellular processes in rat aorta (Jan et al., 1998; Lewis and Spalding, 1998; Geitmann and Cresti 1998; Friedman et al., 1998).

Conclusion

The results of the present study, using combined functional and biochemical *in vitro* approaches, indicated that DMF relaxes pre-contracted isolated rat aortic rings. Such a vasorelaxation was an endothelium-dependent effect, via the NO/sGC/cGMP pathway. This result also suggests that DMF causes a slow influx of extracellular Ca^{2+} release from the intracellular Ca^{2+} stores and an inhibition of Ca^{2+} extruding mechanisms.

ACKNOWLEDGEMENT

Special thanks to CICOPS, Università degli studi di Pavia, Italy, for their research fellowship.

ABBREVIATIONS

DMF, 6,8-Dihydroxy-4'-methoxyflavone; **NO,** nitric oxide; **La^{3+},** lantan 3+; **ATP,** adenosine triphosphste; **pD2,** potent vasorelaxation; **DMSO,** dimethylsulfoxide; **L-NAME,** Nω-nitro-L-arginine methyl esther; **ODQ,** 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one; **$0Ca^{2+}$,** absence of extracellular Ca^{2+} ; **$[Ca^{2+}]_i$,** intracellular Ca^{2+} concentration; **PSS,** physiological salt solution; **HPLC,** high performance liquid chromatography; **NMR,** nuclear magnetic resonance.

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