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Nitric oxide-dependent vasodilation and Ca^{2+} signalling induced by erythrodiol in rat aorta

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ABSTRACT

Objective: To evaluate the pharmacological property of erythrodiol, a natural triterpenoid contained in propolis, as vasodilatory agent, and to determine its mechanism of action.
Methods: Rats aortic rings were isolated and suspended in organ baths, and the effects of erythrodiol 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+} concentration in intact endothelium was opened 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*, 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.
Results: In aortic rings with intact endothelium pre-contracted with norepinephrine (10^{-4} mol/L), the addition of erythrodiol (10^{-8}-10^{-4} mol/L) induced vasorelaxation in a concentration-dependent manner; in endothelium-denuded rings, the relaxant response induced by erythrodiol was almost completely abolished suggesting that vasorelaxation was endothelium-dependent. They had almost no relaxant effect on depolarised or endothelium-denuded aortic segments. The relaxation was significantly attenuated by pre-treatment with the NO synthase inhibitor Nv-nitro-L-arginine-methylester. Erythrodiol (10^{-4} mol/L) was able to significantly increase NOx levels. This effect was completely abolished after removal of the vascular endothelium. Erythrodiol (100 µmol/L) caused a slow, long-lasting increase in intracellular Ca^{2+} concentration. These results further supported the hypothesis that erythrodiol can induce activation of the NO/soluble guanylate cyclase/cyclic guanosine monophosphate pathway, as suggested by functional studies.
Conclusions: The present results suggest that the mechanism of relaxation seems to be mainly mediated by the endothelial production of NO. Such a vasorelaxation was an endothelium-dependent effect, via the NO/soluble guanylate cyclase/cyclic guanosine monophosphate pathway. This result also suggests that erythrodiol causes a slow influx of extracellular Ca^{2+} release from the intracellular Ca^{2+} stores and an inhibition of Ca^{2+} extruding mechanism. It can be concluded that erythrodiol may have interesting therapeutic potential as a new vasodilator drug, for protecting the cardiovascular system.

1. Introduction

Propolis is a generic name of a resinous substance produced by bees. Propolis refers to a whole series of resinous, gums and balsamic substances, of viscous consistency, collected on certain parts (buds and barks primarily) of plants (certain trees mainly) by worker bees, which bring them back to the hive and which add to them some of their own secretions[1,2]. It was used in traditional medicine into 3000 before J.-C.[3]. The Greeks used it for manufacturer of the pomade, and Egyptians for the mummification and to anaesthetize dental decays[1]. It was used by the Arabs for eczemas to attenuate the rheumatic muscular

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pains; in Europe, a propolis/vaseline mixture was used for the asepsis of wounds[4].

Olean-12-ene-3β, 28-diol (erythrodiol) is a compound isolated from propolis originated from Meiganga (Adamawa, Cameroon). To the best of our knowledge, there has been no report on the biological activity of this triterpenoid. The pharmacological effects of erythrodiol have not been studied in detail. Only the therapeutic efficiency of erythrodiol on different experimental models of inflammation has been reported[5,6]. Our interest in this molecule on the cardiovascular system arises from their anti-inflammatory properties mentioned above. Inflammation has been recently linked to the atherogenic process, which also impairs endothelial function, and it is related to vascular reactivity[7]. Endothelium-derived nitric oxide (NO) contributes to cardiovascular homeostasis through its profound effects on blood pressure, vascular remodeling, platelet aggregation, and angiogenesis[8]. It is well known that for some agonists, such as acetylcholine and bradykinin, a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is necessary for NO production[9,10]. However, recent studies indicated that shear stress, isometric contraction, tyrosine phosphatase inhibitors, insulin-like growth factors-1, activated endothelial nitric oxide synthase (eNOS) in isolated vessel preparations or in cultured cells in the absence of a change in $[Ca^{2+}]_i$[11-14]. These Ca^{2+}-insensitive responses are blocked by tyrosine kinase inhibitors. It, thus, appears that eNOS activity is also regulated by a Ca^{2+}-independent mechanism. 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 derived from the oxidation of L-arginine by Ca^{2+}-calmodulin–dependent NOS, an enzyme constitutively expressed in the endothelial cells[15,16]. Furthermore, NOS can be activated by an Akt/PKB, Ca^{2+}-independent pathway[17,18]. Neuronal NOS (nNOS) and eNOS are activated by agonist-induced elevation of $[Ca^{2+}]_i$ with subsequent binding of Ca^{2+}/calmodulin to these enzymes. In contrast, inducible NOS (iNOS) binds to calmodulin with such high affinity that it is already maximally activated at the Ca^{2+} concentrations within resting cells[19]. These different regulatory mechanisms determine the NOS activity in response to various physiological or pathological stimuli, thereby participating in the control of a variety of cell functions[20]. In this study, we attempted to evaluate the possible vasodilatory effects of erythrodiol in rat aorta and the influence of endothelium-derived factors on these actions. The underlying mechanism involved in increasing $[Ca^{2+}]_i$ and vasorelaxant responses was also investigated, by using different experimental protocols.

2. Materials and methods

2.1. Animals

Wistar rats of (300 ± 50) g were used for all experiments. Animals were allowed to acclimate in environmentally controlled quarters with temperature maintained at (22 ± 2) ℃ and lighting at 12 h light/dark cycles for at least a week before being used in experiments. Humidity was maintained at 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.

2.2. Drug administration

The following drugs were used: adenosine triphosphate (ATP), erythrodiol, lantan 3+, dimethylsulfoxide (DMSO), norepinephrine, acetylcholine chloride, atropine sulfate, Nω-nitro-L-arginine methyl esther (L-NAME), indomethacin, L-arginine, and 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions were prepared in distilled water and kept at -4 ℃. Erythrodiol 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 DMSO had no effect when tested in control preparations (data not shown).

2.3. Chemicals solutions

The composition of the Tyrode's solution used was 158.30 mmol/L NaCl, 4.00 mmol/L KCl, 2.00 mmol/L $CaCl_2$, 1.05 mmol/L $MgCl_2$, 0.42 mmol/L NaH_2PO_4, 10.00 mmol/L $NaHCO_3$, and 5.60 mmol/L glucose. The physiological salt solution (PSS) had the following composition (mmol/L): 150.0 NaCl, 6.0 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 10.0 glucose, 10.0 2-hydroxyethyl, at pH 7.4. In Ca^{2+}-free solution, Ca^{2+} was substituted with 2.0 mmol/L NaCl, and 0.5 mmol/L ethylene glycol tetraacetic acid 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, Netherlands). All other chemicals were purchased from Sigma Chemical Co. (USA). 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.

2.4. Propolis collection

Propolis raw material was collected from Ngaoundal (2009; Adamawa region; Cameroon), agro-ecological zone. Propolis sample was identified by professor Fernand-Nestor Tchuenguem Fohouo, Entomologist and beekeeper at the University of Ngaoundéré.

2.5. Extraction and isolation of the molecule

Propolis raw material was powdered (950 g) and extracted three times (6 L × 3) in methanol (MeOH) at room temperature for one week (48 h × 3). After each filtration, the solvent was evaporated under reduced pressure with a rotary evaporator (Büch, 461). The brown crude methanolic extract (575.2 g) was obtained and

successively partitioned with n-hexane and ethyl acetate (EtOAc) solvents to yield 255.1 and 125.6 g respectively. The remaining dry methanolic residue extract was 165.8 g. The extraction yield was calculated (Table 1).

Table 1
Extraction yield of crude extracts.

Extract code	Total weight of crude extract (g)	Extraction yield (%)
PMCE	575.2	60.5
PHEn	255.1	26.8
PEAEn	125.6	13.2
PMRE	165.8	17.4
Total extraction yield (%)		57.4
Lost mass of crude extract after partition	28.7	3.1

PMCE: Propolis methanol crude extract; PHEn: Propolis n-hexane extract; PEAEn: Propolis ethyl acetate extract; PMRE: Propolis methanolic residue extract.

A part of dry methanolic residue extract (PMRE, 150 g) was subjected to silica gel column chromatography (Ø 0.063-0.200 mm, 650 g) and eluted with the mixture n-hexane-CH_2Cl_2 and CH_2Cl_2-MeOH in order of increasing polarity (0%-100%) to yield a total of 143 fractions of 250 ml each. These fractions were combined on the basis of TLC analysis into ten major fractions (A-J). Fraction A was subjected to silica gel column chromatographic purification (Ø 0.063-0.200 mm, 500 g) using the mixtures n-hexane-EtOAc and EtOAc-MeOH with gradient polarity (0%-100%) as eluents. Sub-fractions were combined on the basis of TLC analysis into sixteen serial profiles (A'-P'). A white powder was formed in F' (69-70) series from the mixture n-hexane-EtOAc (9:1). After filtration and recrystallization, 151 mg of the white crystal was obtained and indexed PS26, then subjected to spectral analyses.

2.6. Spectral characterization of PS26

The ^{13}C NMR and DEPT spectra indicated the presence of 30 carbon atoms, suggesting a molecular formula $C_{30}H_{50}O_2$ with 6 unsaturations. The 30 carbon atoms (^{13}C NMR), from which 7 of them are attributed to methyl groups at δC: 28.0, 15.5, 15.5, 16.7, 25.9, 33.1, 23.5; one for oxymethine at δC 79.0 and 04 methine groups at δC: 55.1, 47.5, 122.3, 42.3; 11 methylene groups at δC: 38.5, 27.2, 18.3, 32.5, 23.5, 25.5, 22.0, 46.4, 34.0, 30.9, 69.7 as well as the signals for quaternary carbons at δC: 38.7, 39.7, 36.9, 144.2, 41.7, 36.9, 31.0. In addition, the oleanane skeleton is confirmed by the presence of two characteristic signals at δC = 122.3 and δC = 144.2 corresponding to C-12 and C-13 carbon atoms respectively.

^1H NMR spectrum displayed signals of a set of seven methyl protons groups at δH: 0.74, 0.89, 1.14, 1.62, 1.56, 1.78, 1.97. One thus noted also the presence of two doublets and one resounded at 3.5 ppm corresponding to the geminal proton of -CH_2OH group. The chemical shift of this proton indicated that it was about an equatorial proton, OH group was thus in position (for biogenetic considerations) and the other at 3.12 ppm. One also noted the presence of a triplet at 5.12 ppm probably indicating the olefinic proton. Those thus led to the identification of PS26 as erythrodiol also known as erythrodiol (CAS NO. 545-48-2) (Figure 1).

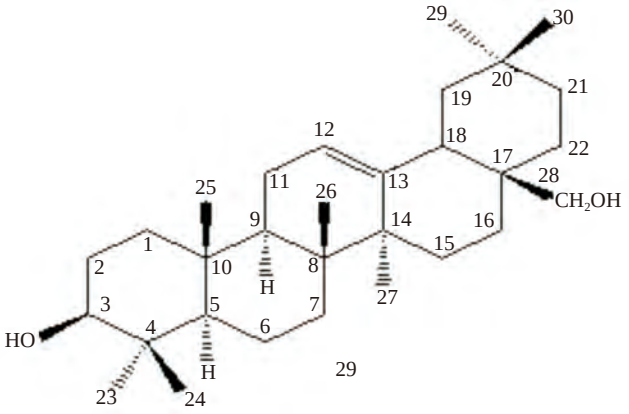

Figure 1. Structure of erythrodiol.

2.7. Preparation of isolated superior rat aorta rings

Wistar rats were sacrificed by stunning and bleeding. The thoracic and abdominal aortas were dissected out and perfused with 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 h; the rings were removed and stored in PSS for 30 min, at room temperature of 22-24 ˚C. When appropriate, the endothelium was removed by gently rubbing the intimal surface of the vessels. Rings (1-2 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 kept 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[21]. 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 μmol/L) to induce more than 90% relaxation of vessels pre-contracted with norepinephrine (10 μmol/L) and the absence, less than 10% of relaxation to acetylcholine was taken as an evidence that the vessel segments were functionally denuded of endothelium[22]. 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 μmol/L), NOS inhibitor; L-NAME (100 μmol/L) plus L-arginine (1 mmol/L), the endogenous substrate of NOS; ODQ (10 μmol/L), a soluble guanylyl cyclase (sGC) inhibitor; or indomethacin (10 μmol/L), a cyclo-oxygenase (COX) inhibitor, plus atropine (1 nmol/L), a non-selective muscarinic-receptor antagonist[23-27], used separately. These inhibitors were added 30 min before the application of norepinephrine. In the tonic phase of the second contraction, erythrodiol (10^{-7}-10^{-4} mol/L) was cumulatively added to the preparations. Inhibition was calculated by comparing the response of erythrodiol before and after the addition of the inhibitors or antagonists.

2.8. Determination of NO level

The rat aorta was removed as described above, and rings of 1-2 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 nitrogen oxides (NOx) and the ring weight. The total amount of NOx in the medium was determined by using the purge system of Sievers instruments (model NOA 280i, Boulder, CO, USA)[28]. A saturated solution of vanadium chloride (VCl 3) in 1 mol/L HCl was added to the nitrogen-bubbled purge vessel fitted with a coldwater condenser and a water jacket to allow heating of the reagent to 90 °C by using a circulating bath. HCl vapors were removed by a gas bubbler containing sodium hydroxide (1 mol/L). Flow of gas into the detector was controlled by a needle valve adjusted to yield a constant pressure. When the detector signal was 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 μmol/L) for 20 min. After this period, in each hole, 10 μmol/L of acetylcholine was administered as a positive control and the molecule as negative control. After half an hour, the center of each hole was recovered and used for the determination of NOx, as described above. To investigate whether NO production was Ca^{2+}-dependent, erythrodiol 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 applying La^{3+} (100 μmol/L) in Ca^{2+}-free solution for 30 min, and then washing the ring by extracellular Ca^{2+}-free solution and applying erythrodiol in extracellular Ca^{2+}-free solution; applying ATP 300 μmol/L 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 ethylene glycol tetraacetic acid (final concentration 0.5 mmol/L (pH 7.4). To study the effects of erythrodiol on Ca^{2+} influx from voltage-dependent Ca^{2+} channel, the vascular smooth muscle cells were challenged with KCl (80 mmol/L) in the presence of erythrodiol (10^{-8}-10^{-4} mol/L) and the changes in intracellular Ca^{2+} were recorded.

2.9. Assessment of effect of erythrodiol on blood pressure

Wistar rats were anesthetized by intraperitoneal administration of sodium pentobarbital (40 mg/kg) and kept on a heating pad for the maintenance of body temperature at (37 ± 1) °C. The windpipe was intubated with a polyethylene tube (3 mm diameter) in order to facilitate spontaneous respiration. The right femoral artery and vein were cannulated using P.E.-50 tubing (Clay Adams, Parsippany, NJ) for the monitoring of pulse pressure and mean arterial pressure and for *i.v.* bolus administration of erythrodiol. The registering apparatus of blood pressure was a multispeed 2 400 polygraph (valley view) via pressure transducer (Viggo-spectramed, Oxnard, CA). Changes in MAP after erythrodiol administration were compared with those after the injection of the same volume of vehicle.

2.10. Intracellular $[Ca^{2+}]_i$ evaluation

The technique used to evaluate changes in intracellular $[Ca^{2+}]_i$ in intact endothelium has previously been described[29,30]. The aortic ring was opened and loaded with 16 μmol Fura-2/AM for 60 min at room temperature and 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 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 (21-23 °C).

2.11. Data analysis

Mean values are presented as mean ± SE 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 ANOVA, using Origin Graph, (Microcal Origin 6.0) software version 6.0. Tracings shown in the figures were single cell recording. The EC_{50} 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 measured by the Fit Linear regression. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of Erythrodiol on mean arterial pressure

The average AMP before erythrodiol treatment in 10 anesthetized rats was (115 ± 3) mmHg. Mean body weight for this experiment was (288 ± 6) g. Figure 2 demonstrates the dose dependence of the effect of erythrodiol (0.5-2 mg/kg) on AMP. The antihypertensive effects occurred after administration of the erythrodiol, and reached an optimal activity 45-60 min later. At 2 mg/kg of erythrodiol, the initial AMP (artery mean pressure) of (107.35 ± 2.88) mmHg decreased to (81.12 ± 3.24) mmHg, thus a reduction of 24.43%. The pressure gradually increased to the

initial values before a new decrease and stabilization at around (87.21 ± 2.12) mmHg, for a 36.52% reduction of the AMP. Erythrodiol provoked an immediate and significant decrease in AMP at 2 mg/kg that passed from (117.93 ± 3.45) mmHg to (82.22 ± 5.32) mmHg, thus a 29.43% reduction (Figure 2). The pressure increased up to the 20th min, then decreased and was maintained at values lower than the initial pressure. The hypotensive maximal effect (Emax = 42%) was observed 1 h after administration of 2 mg/kg dose of erythrodiol. Atropine effect on the hypotensive activity of erythrodiol was then assessed. Atropine was administrated 3 min before the erythrodiol (2 mg/kg) as shown in Figure 3. The pretreatment of animals with atropine (2 mg/kg) provoked a significant inhibition of the late hypotensive response induced by erythrodiol.

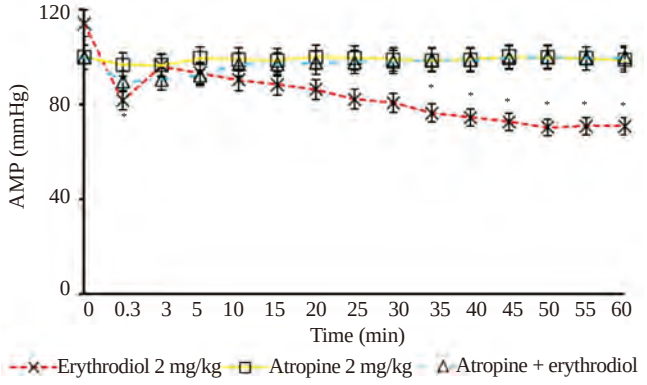

Figure 2. Effects of erythrodiol on the arterial mean pressure.
Data were expressed as mean ± SEM (n = 5). *: P < 0.05.

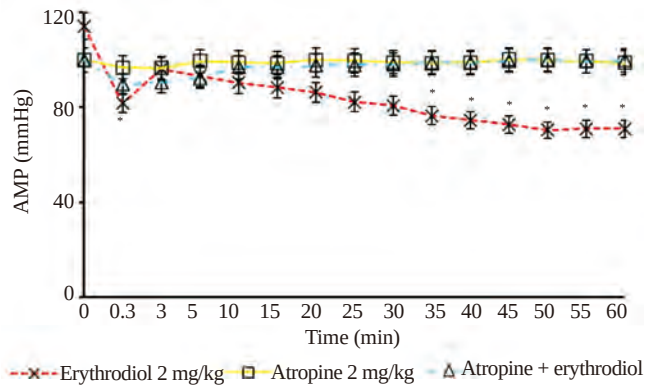

Figure 3. Effects of atropine on hypotensive activity of erythrodiol on the arterial mean pressure.
Data were expressed as mean ± SEM (n = 5). *: P < 0.05.

3.1. Relaxant activity of erythrodiol

3.1.1. Norepinephrine-induced contractions

Rat aortic strips precontracted with norepinephrine (10^{-4} mol/L) were relaxed dose-dependently by erythrodiol (Figure 4). Exogenous additions of graded concentrations of erythrodiol (10^{-8}-10^{-4} mol/L) to aortic ring strips pre-contracted with norepinephrine (10^{-4} mol/L), evoked concentration-dependent relaxation responses of the muscle strips (Figure 4). The relaxation of aorta muscle precontracted with norepinephrine increased with increasing doses of erythrodiol (10^{-8}-10^{-4} mol/L), from 32.39% at the dose 10^{-8} mol/L to 80.22% (Emax = 88%) at 10^{-4} mol/L on intact aorta and from 8.63% to 18.49% at respective doses on denuded aortic strips. The responses of erythrodiol in endothelium-denuded aortic rings (EC_{50} = 13.36 ± 0.67), however, were significantly lower than those with endothelium intact rings (EC_{50} = 66.92 ± 2.33) (Figure 4). Endothelium-intact preparations were therefore used to study the roles of endothelial-derived relaxing factor (EDRF), calcium and potassium channels in the vasorelaxant effects of erythrodiol. The vasorelaxant effects of erythrodiol in endothelium intact aortic rings were significantly reduced by specific inhibitors of EDRF (L-NAME) (Figure 5). In the presence of the NOS inhibitor L-NAME (100 μmol/L), the relaxation induced by erythrodiol in rings with or without endothelium was similar to that induced in endothelium-denuded rings (Figure 5). The inhibitory effect of L-NAME (100 μmol/L) was completely reversed by the addition of the biological substrate of NO synthase L-arginine (1 mmol/L) (EC_{50} = 44.02 ± 1.21) (Figure 5). In the presence of ODQ (10 μmol/L), an inhibitor of the soluble guanylyl cyclase, the relaxation induced by erythrodiol (10^{-8}-10^{-4} mol/L) was almost completely abolished (EC_{50} = 6.15 ± 0.21) (Figure 5). We also investigated the contribution of relaxant arachidonic acid derivatives and activation of the muscarinic receptors. In rings pre-incubated with indomethacin (1 μmol/L) plus atropine (1 nmol/L) (EC_{50} = 65.12 ± 2.16) (Figure 6), the relaxation induced by erythrodiol was not significantly different from the relaxation induced in endothelium-intact rings. These results suggested that the vasorelaxant effect of erythrodiol was in part dependant on EDRF. The effect of erythrodiol on norepinephrine-induced contractions was not altered in the absence of the functional endothelium when compared to endothelium-intact rings.

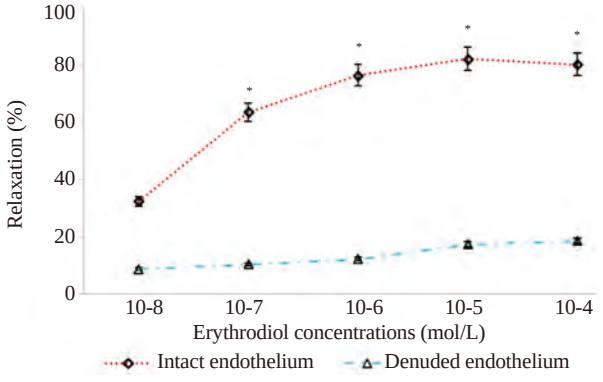

Figure 4. Relaxation induced by erythrodiol in endothelium-intact or endothelium-denuded rat aortic rings.
Values were expressed as mean ± SEM. *: P < 0.05.

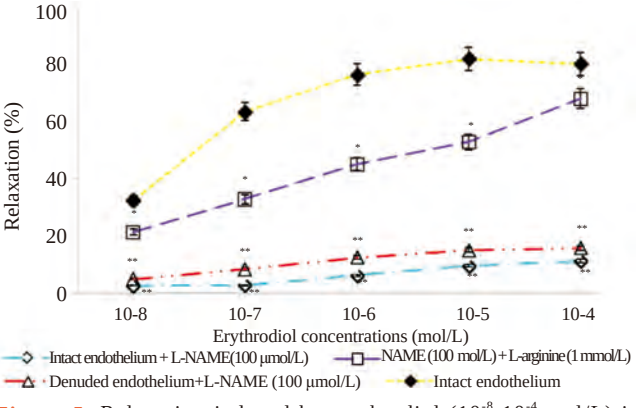

Figure 5. Relaxation induced by erythrodiol (10^{-8}-10^{-4} mol/L) in endothelium-intact rings (n = 5), endothelium-denuded rings, after pre-treatment with L-NAME (100 μmol/L; n = 5), and endothelium-intact rings after pre-treatment with L-NAME (100 μmol/L) plus L-arginine (1 mmol/L) (n = 5).
Values were expressed as mean ± SEM. *: P < 0.05, **: P < 0.01.

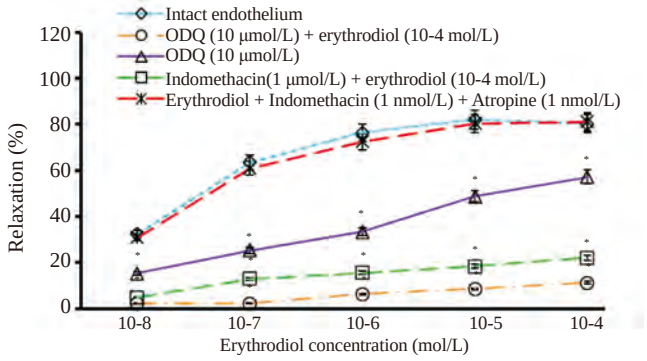

Figure 6. Relaxation induced by erythrodiol (10^{-8}-10^{-4} mol/L) in endothelium-intact aortic rings, after pre-treatment with ODQ (10 µmol/L), and after (n = 6) pre-treatment with indomethacin (1 µM) plus atropine (1 nmol/L) (n = 5).
Values were expressed as mean ± SEM. *: $P < 0.05$.

3.1.2. KCl-induced contractions

To evaluate the role of potassium channels in the vasorelaxant effects of erythrodiol, studies were conducted on endothelium-intact aortic rings pre-contracted with low KCl concentrations (20 mmol/L). At final concentrations between 10^{-8} mol/L and 10^{-4} mol/L, erythrodiol did no significantly affect the baseline tension in aortic strips. Erythrodiol induced significant and concentration-dependent vasorelaxations in these aortic rings. Pretreatment with glibenclamide before inducing contraction with low KCl did not modify the vasorelaxant effect of erythrodiol (Figure 7). To evaluate the role of calcium channels in the vasorelaxant effects of erythrodiol, experiments were conducted in endothelium intact aortic rings pre-contracted with high KCl concentrations (80 mmol/L). The vasorelaxant effects of graded erythrodiol concentrations were not statistically different in these aortic rings pre-contracted with low or high KCl concentrations (Figure 7). Aortic ring preparations contracted with 80 mmol/L KCl was used to study compounds with Ca^{2+} entry-blocking properties. The maximum relaxation at the highest dose of erythrodiol when aortic muscle was pre-contracted with KCl were 85% and 78%, respectively, on endothelium-intact arteries and endothelium denuded arteries. There was a significant difference between effects on intact and denuded aortic strips in all concentration tested. Acetylcholine (10^{-5} mol/L), a drug with known vascular smooth muscle relaxant properties, used as the standard drug produced a relaxation of intact aorta (36%). Pretreatment of intact aortic strips with the specific inhibitor of ATP-dependent K^+ channels, glibenclarnide (10^{-6} mol/L), had no significant effects on erythrodiol induced relaxation of aortic strips. In addition, it was observed (data not shown) that incubation of the non pre-contracted rat aortic strips with erythrodiol (10^{-4} mol/L) inhibited the contractile response induced by KCl (60 mmol/L) by 62%.

3.2. NO production

Figure 8 shows the effects of erythrodiol (10^{-4} mol/L) and acetylcholine (10^{-5} mol/L) on NOx level in rat aorta rings pre-contracted with norepinephrine (10^{-4} mol/L), in aortic rings with intact endothelium or removed endothelium. Erythrodiol (10^{-4} mol/L) was able to significantly increase NOx levels in isolated rat aorta rings. This effect was completely abolished after removal of the vascular endothelium. Acetylcholine (10^{-5} mol/L), used as positive control for NO production induced an increase in NO-levels about 50% of the one induced by 10^{-4} mol/L erythrodiol when submitted to the same experimental conditions.

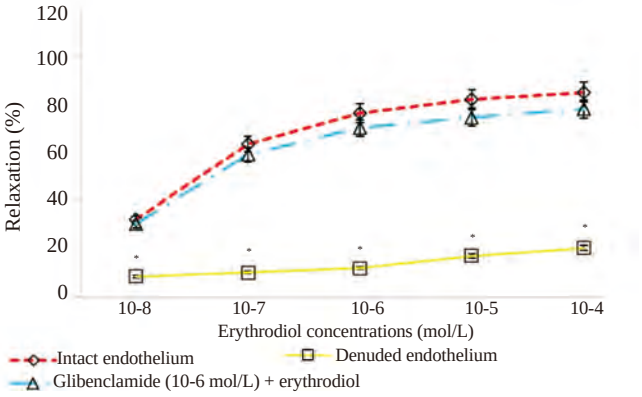

Figure 7. Relaxation induced by erythrodiol (10^{-8}-10^{-4} mol/L) in endothelium-intact aortic rings, after pre-contracting with KCl (80 mmol/L), and after pre-treatment with glibenclamide (10-6 mmol/L) (n = 5).
Values were expressed as mean ± SEM. *: $P < 0.05$.

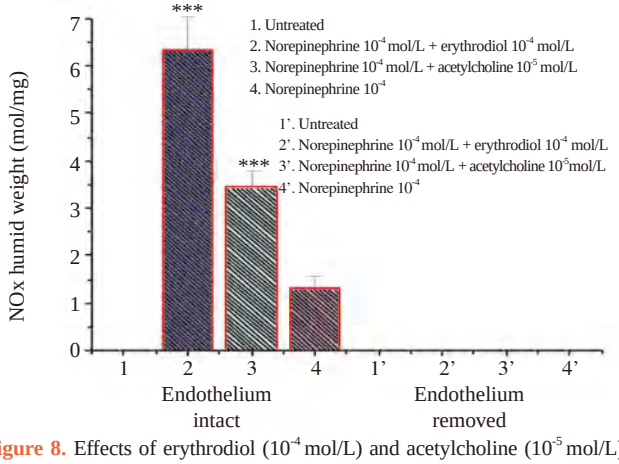

Figure 8. Effects of erythrodiol (10^{-4} mol/L) and acetylcholine (10^{-5} mol/L) on NOx level in rat aorta rings pre-contracted with norepinephrine (10^{-4} mol/L), in aortic rings with intact endothelium or removed endothelium (n = 5)
Values were expressed as mean ± SEM. ***: $P < 0.001$.

3.3. Effects of Erythrodiol on intracellular calcium in vascular smooth muscle cells

KCl (80 mmol/L) in Ca^{2+}-free solution, was depolarized the cell membrane of aortic smooth muscle and voltage-dependent Ca^{2+}-channel were activated. The failure of KCl to produce vasoconstriction in the aortic rings in the absence of extracellular Ca^{2+} proved the lack of Ca^{2+} entry (data not shown). The increase contraction of the rat aorta, apparently caused by Ca^{2+} entering the depolarized cell through voltage-dependent Ca^{2+}- channel (Figure 9). The maximal tension attained at 4 mmol/L Ca^{2+} was (1.89 ± 0.22) g in the presence of KCl. The IC_{50} value was calculated to be (7.11 ± 0.14) µmol/L at Ca^{2+} concentration of 4 mol/L. When the aortic ring was treated with erythrodiol at 10^{-8}-10^{-4} mol/L, 10 min before KCl, the KCl-induced concentration was attenuated in a concentration-dependent manner, suggesting that Ca^{2+} influx through voltage-dependent Ca^{2+}-channel was probably inhibited by erythrodiol.

3.4. Effects of erythrodiol in Ca^{2+} signalling

The introduction in the incubation medium of erythrodiol in in situ endothelium of aortic rings 20 min before the higher magnitude response produced by ATP and 100s before the addition of erythrodiol did not provoke any significant modifications of the amplitude effects of erythrodiol (data no shown). Erythrodiol (100 µmol/L) caused a slow, long-lasting increase in $[Ca^{2+}]_i$ (Figure 10). The magnitude 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 erythrodiol (Figure 11), suggesting that erythrodiol could also 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 (1.19, correlation coefficient = 0.94; n = 8) (Figure 12) much smaller than control values (7.27, correlation coefficient = 0.92; n = 9), suggesting that Ca^{2+} influx was involved. In the presence of La^{3+}, the higher slope due to erythrodiol (7.87, correlation coefficient = 0.96; n = 8) was completely abolished as shown in Figure 13, suggesting that erythrodiol-induced intracellular influx Ca^{2+} involves the participation of channels calcium. These results further support the hypothesis that erythrodiol can induce activation of the NO/sGC/cGMP pathway, as suggested by functional studies.

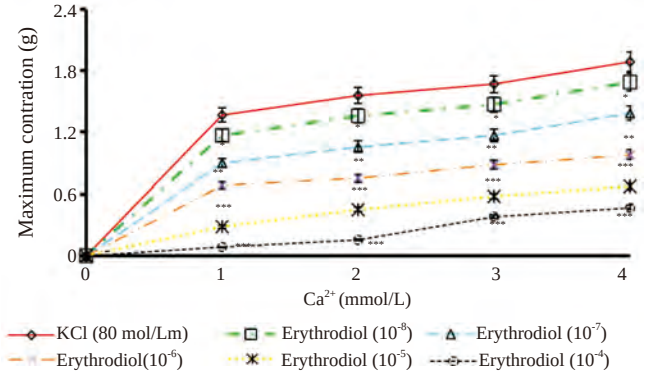

Figure 9. Effects of the cumulative addition of Ca^{2+} (10^{-4} mnol/L) caused a stepwise increase of the contraction of the rat aorta.
Inhibitory effect of erythrodiol (10^{-8}-10^{-4} mol/L) on the contraction dependent on extracellular Ca^{2+} influx induced by KCl (80 mmol/L) in Ca^{2+}-free solution of rats endothelium-denuded rats aortic rings (n = 5).
Values were expressed as mean ± SEM. *: $P < 0.05$; **: $P < 0.01$ ***: $P < 0.001$.

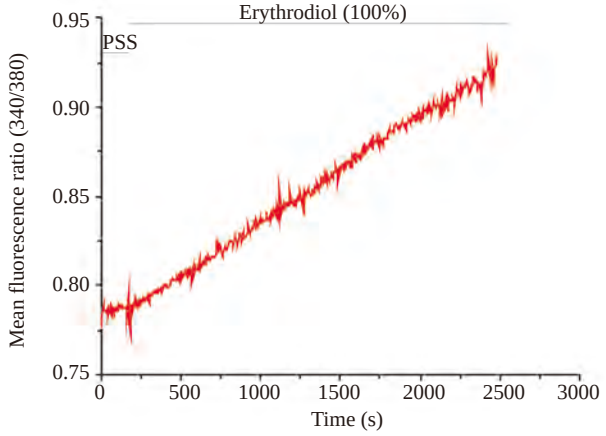

Figure 10. Effect of erythrodiol (100 µmol/L) on the intracellular Ca^{2+} concentration of aorting ring endothelial cells (single cell tracing).

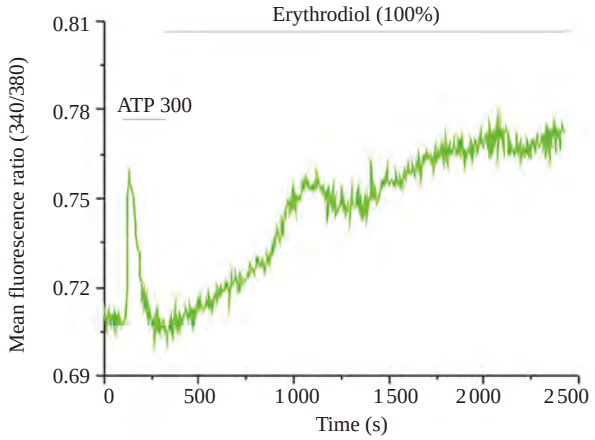

Figure 11. Effect of ATP (300 µmol/L) and erythrodiol (100 µmol/L) on the intracellular Ca^{2+} concentration of aorting ring endothelial cells (single cell tracing).

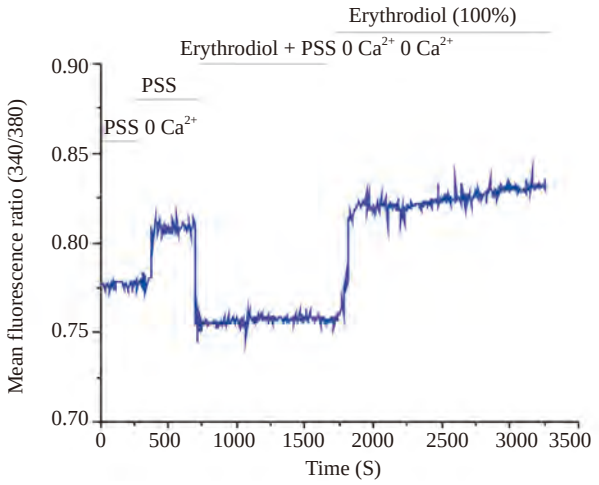

Figure 12. Effect of erythrodiol (100 µmol/L)on the intracellular Ca^{2+} concentration of aorting ring endothelial cells in the presence (PSS) and absence of extracellular Ca^{2+} (0 Ca^{2+}) (single cell tracing).

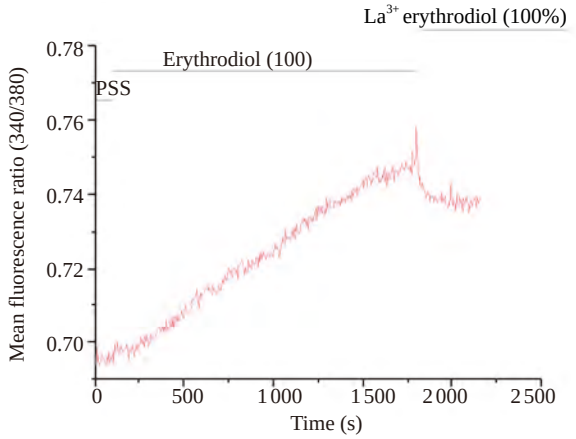

Figure 13. Effect of erythrodiol (100 µmol/L) on responses to the influx of extracellular Ca^{2+} after (n = 8) pre-treatment of the rings with La^{3+} (100 µmol/L).

4. Discussion

The findings of this study indicate that erythrodiol possesses hypotensive properties. The *in vivo* reduction in blood pressure by erythrodiol occurred without significant alterations in heart rate, possibly suggesting that the *in vitro* cardiovascular effects of

erythrodiol significantly contributed to its hypotensive effects. The present study investigates the vasorelaxant activity of erythrodiol in rat aorta. We have studied the ability of the compound to induce a relaxation of contractions caused by different agonists, KCl or norepinephrine, in isolated rat aorta. In this paper, we have shown that erythrodiol, a triterpenoids isolated from propolis from meiganga (Cameroon) induces a strong, dose-dependent relaxation of the rat aortic rings pre-contracted with norepinephrine. It is has been reported that high KCl^+ concentrations cause contractions in vascular smooth muscle by depolarising cell membranes and by increasing the influx of Ca^{2+} through long-lasting voltage dependent channels[31]. In this way, the absence of relaxation in 80 mmol/L-KCl-evoked contractions might probably remove either the influence of membrane hyperpolarisation or the contribution of the blockage of Ca entry through voltage-stimulated Ca^{2+} channels to the relaxant responses to erythrodiol. Pretreatment with glibenclamide before inducing contraction with low KCl did not modify the vasorelaxant effect of erythrodiol. Pretreatment with glibenclamide before inducing contraction with low KCl did not modify the vasorelaxant effect of erythrodiol. The results obtained in this study also suggest that the erythrodiol evoked vasorelaxations in aortic ring preparations were mediated through both endothelium-derived relaxing factor-dependent and -independent mechanisms. Indeed, graded concentrations of erythrodiol elicited dose dependent vasorelaxations in endothelium-intact and -denuded aortic ring preparations, although the erythrodiol vasodilatory effect was less in the latter protocol. Furthermore, the vasorelaxations produced by erythrodiol in endothelium-intact aortic rings were pharmacologically modulated by L-NAME, a non-selective nitric oxide synthase inhibitor[32], suggesting the involvement of endothelial synthesised NO. The vascular endothelium has been shown by Furchgott and Zawadzki[33] to be of crucial importance in the relaxation of blood vessels in response to acetylcholine and some other naturally occurring vasodilator substances[34]. Although NO appears to be the major vasodilator released by endothelial cells, other substances may also play a role, including prostacyclin and the endothelium-derived hyperpolarising factor. These endothelial-relaxing factors contribute to the protective role of the endothelium[35]. It has become clear that endothelial cells not only release relaxing factors but also produce contracting substances such as endothelin, thromboxane A2 and prostaglandin H_2[26] as well as reactive oxygen species[36]. Furthermore, erythrodiol relaxing activity is fully dependent on endothelial cells, via the NO–cGMP pathway. Indeed, mechanical removal of endothelium abolished the relaxant response induced by erythrodiol, suggesting that erythrodiol activates an endothelium-dependent mechanism. To elucidate a mechanism, we first evidenced that inhibition of NO synthesis by the L-arginine analogue L-NAME abolished the vasorelaxation induced by erythrodiol. Furthermore, in rings pre-incubated with L-NAME plus L-arginine, the vasorelaxante response by erythrodiol was completely reversed, suggesting that the erythrodiol effect is mediated by a mechanism involving endothelium-derived NO.

Endothelial NO synthesis is regulated by a variety of stimuli that trigger release of multiple vasoactive substances, including NOS[34,37-39]. 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)[17,18]. NO activates vascular Ca^{2+}-activated channels directly[40] and/or through cyclic GMP dependent mechanisms [41], and causes smooth muscle relaxation[42,43,44]. To determine if erythrodiol -induced relaxation involves the participation of a cGMP pathway the preparations were pre-treated with ODQ, a soluble guanylyl cyclase inhibitor[25]. In these conditions, the vasorelaxation induced by erythrodiol was almost completely abolished. These results suggest that erythrodiol -elicited vasorelaxant effects on the vascular smooth muscles were mediated via NO and/or cGMP and cyclooxygenase pathways. It is now generally accepted that relaxation of vascular smooth muscles involves the lowering of intracellular calcium mediated by cGMP-dependent or -independent pathways. 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[33,45,46] or muscarinic receptor activation. To investigate the participation of theses pathways, we pre-treated the vessels with indomethacin (10 μmol/L) plus atropine (1 nmol/L). In this condition, erythrodiol's effects were similar to that obtained in control conditions, suggesting that muscarinic-receptor activation or COX-derived products are not involved. The results clearly show that erythrodiol are able to relax, in a concentration-dependent manner, the contractions induced norepinephrine and KCl in rat aortic rings with functional endothelium.

To further confirm such a hypothesis, a biochemical assay using a very sensitive technique for NOx analysis was performed[47,28]. 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, have been frequently measured to determine NO production[48]. In endothelium-intact aorta rings pre-contracted with norepinephrine, erythrodiol (10^{-4} mol/L) and acetylcholine (10^{-5} mol/L, used as positive control) significantly increased NOx levels. In response to 10^{-4} mol/L erythrodiol, NOx levels increased to virtually the same level as that reached after 10^{-5} mol/L acetylcholine. Inversely, in rings in which the vascular endothelium was removed, no effect of erythrodiol on NOx levels was observed, excluding a possible NO-donating effect induced by the compound.

In this study, pretreatment with erythrodiol suppressed in a concentration-dependent manner, the aortic contractile response to high KCl. the maximun inhibition produced by erythrodiol at 10^{-4} mol/L was about 88%. When erythrodiol was cumulatively added during the tonic contraction induced by high KCl, it exerted 100% vasorelaxation. Similar results were also obtained as the aortic preparation were challenged with the voltage-dependent Ca^{2+} channel activator. These observation suggests that erythrodiol might intefere with these Ca^{2+} channels in the aortic smooth muscle, possibly resulting in the decrease of Ca^{2+} influx and contraction. Furthermore, in Ca^{2+}-depleted and high KCl medium, the cell membrane of aortic smooth muscle was depolarized and to lack of extracellular Ca^{2+}. Addition of Ca^{2+} produced sustained contraction that was produced by the Ca^{2+} influx through voltage-dependent Ca^{2+} channel. Pre-incubation with erythrodiol could effectively antagonize, in a concentration-dependant manner, Ca^{2+} induced contraction, impying that erythrodiol probably blocked Ca^{2+} influx through voltage-dependent Ca^{2+} channel in isolated aortic smooth muscle cells. A plausible reason is probably that much higher concentration of Ca^{2+} could be needed to achieve the maximal contraction because Ca^{2+} in high concentration can be auto-inhibitory, thus decrease the permeability of the cell membrane for Ca^{2+}.

It is well known that activation of constitutive endothelial NOS may be Ca^{2+} dependent. Erythrodiol caused a slow, long lasting increase in the aortic endothelial cells $[Ca^{2+}]i$. 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 16% of the slope in normal solution) . This result suggests that erythrodiol

causes a slow influx of extracellular Ca^{2+}. Release from the intracellular Ca^{2+} stores and an inhibition of Ca^{2+} extruding mechanisms. The magnitude 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 erythrodiol, suggesting that also erythrodiol could be able to evoke a Ca^{2+}-dependent NO synthesis. Ca^{2+} is known as an important regulatory element for many cellular processes; it acts either directly as a 2nd messenger, for the maximum activation of other enzymes in the signal cascade[49,50]. erythrodiol (10^{-4} mol/L), administrated in Ca^{2+} free medium did not increase intracellular influx Ca^{2+}. This suggest that erythrodiol would act on the calcium channels by stimulating their openings, thus allowing a massive entry of Ca^{2+} into the cell.

To determine if erythrodiol -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[51], inhibited higher amplitude due to erythrodiol -induced Ca^{2+} release. 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 μmol/L) has been extensively used to inhibit Ca^{2+} influx to investigate various Ca^{2+}-dependent cellular processes in rat aorta[52-55].

This study has provided the mechanistic basis for the use of erythrodiol in the management of hypertension and an endothelium-dependent vasorelaxation in rat aorta. These preliminary results suggest that the mechanism of relaxation seems to be mainly mediated by the endothelial production of NO; such a vasorelaxation was an endothelium-dependent effect, via the NO/sGC/cGMP pathway. This result also suggests that erythrodiol causes a slow influx of extracellular Ca^{2+}. Release from the intracellular Ca^{2+} stores and an inhibition of Ca^{2+} extruding mechanisms. The findings may be helpful in the development of an antihypertensive agent from erythrodiol.

Conflict of interest statement

We declare that we have no conflict of interest.

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