

‘ENDOTHELIUM-INDEPENDENT AND ENDOTHELIUM-DEPENDENT VASORELAXATION BY A DICHLOROMETHANE FRACTION FROM *ANOGEISSUS LEIOCARPUS* (DC) GUILL. ET PERR. (COMBRETACEAE): POSSIBLE INVOLVEMENT OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE INHIBITION.

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Abstract

Many traditional medicinal herbs from Burkina Faso are used to treat arterial hypertension (HTA). Among them, *Anogeissus leiocarpus* (*A. Leiocarpus*) which is well known and widely used in Burkina traditional medicine. Herein we assess the effects of dichloromethane fraction from *A. leiocarpus* stem bark (*ALF*), selected as the most active on cyclic nucleotide phosphodiesterases (PDEs) and characterized its specificity towards purified vascular PDE1 to PDE5 isoenzymes and study its effects on a vascular model. *ALF* potently and preferentially inhibits ($IC_{50}=1.6 \pm 0.6 \mu\text{g/mL}$) the calmodulin-dependent phosphodiesterase PDE1, being mainly present in vascular smooth muscle and preferentially hydrolyses cGMP. In the same range ($IC_{50}=2.8 \pm 0.2 \mu\text{g/mL}$) *ALF* inhibits PDE2, a cGMP-activated enzyme that is only present in endothelial cells and hydrolyses both cAMP and cGMP. PDE5, which specifically hydrolyses cGMP and which mainly contributes to cGMP hydrolysis is also potently inhibited by *ALF* ($IC_{50}=7.6 \pm 3.5 \mu\text{g/mL}$). The potencies of *ALF* on cAMP hydrolyzing isoenzymes was lesser, being more effective on PDE4 ($IC_{50}=17.6 \pm 3.5 \mu\text{g/mL}$) than on PDE3 ($60.9 \pm 1.8 \mu\text{g/mL}$). Since the major effect of *ALF* were against cGMP hydrolysis and since cGMP is implicated in endothelium-dependent relaxation, the endothelium-dependent vasorelaxation was studied on isolated porcine coronary arteries rings pre-contracted with U46619. The endothelium-dependent vasorelaxation is significantly inhibited by N^o-nitro-L-arginine (LNA 300 $\mu\text{mol/L}$, an inhibitor of endothelial NO synthase), but not affected by charybdotoxin (CTX, 100nM) plus apamin (APA, 100nM) (two inhibitors of EDHF-mediated responses). The combination of 4-aminopyridine (4-AP, 1 mmol/L, inhibitor of voltage-dependent potassium channels, K_v) plus baryum (Ba²⁺, 30 $\mu\text{mol/L}$, inhibitor of the potassium channels with entering correction, K_{ir}) plus ouabain (3 $\mu\text{mol/L}$, inhibitor of ATPase Na⁺/K⁺ channels) partially inhibits endothelium-independent vasorelaxant effect. This endothelium-independent relaxant effect was also sensitive to combination of 1H-[1,2,4]-oxadiazole-[4,3- α]-quinoxalin-1-one (ODQ, 10 μM , soluble guanylyl cyclase inhibitor) and N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide dihydrochloride (H89, 100 nM, Protein Kinase A inhibitor). Taken together, these results indicate that *ALF* is a powerful vasodilator modulated by the formation of NO from endothelium, but also act by directly relaxing the vascular smooth muscle cells, by inhibiting cGMP hydrolyzing PDEs (PDE1, PDE2 and PDE5) and to a lesser extend on cAMP degradation (PDE3 and PDE4), cAMP and cGMP being second messengers involved in vascular relaxation.

Key words: *Anogeissus leiocarpus*, coronary artery, Vasorelaxation, Endothelium, Phosphodiesterase inhibition.

Introduction

According to the World Health Organization (OMS., 2002), more than 80% of the African populations have recourse to traditional medicine and pharmacopeia for the needs of primary health. In Burkina Faso, following the example of other developing countries, medicinal plants are used in the treatment of various pathologies among which the arterial hypertension (Nacoulma /Ouédraogo, 1996; Yao, 2005). Among these plants, *A. leiocarpus* (D.C.) Guill. and Perr. (Combretaceae) is widely used for some of its properties such as pest-destroying (Okpekon et al. 2004), trypanocides (Shuaibu et al. 2008) and antifungal (Batawila et al. 2005). Recently, antibacterial activity of terpenoidal fractions from *A. leiocarpus* have been shown (Mann et al., 2007). We have demonstrated an anti-HTA property of the aqueous extract of *A. leiocarpus* (Belemnaba, 2007., Ouédraogo et al., 2008). Since, it was demonstrated that some plant extracts inhibit cyclic nucleotide phosphodiesterase (PDE) isoenzymes (Lobstein et al. 2002) and that (PDE1 to PDE5) play a major role in vascular contraction (Lobstein et al. 2002; Lugnier 2006). So this study evaluated the effects of the dichloromethanolic fraction (*ALF*) of *A. leiocarpus* stem bark on the activity of purified vascular PDE isoenzymes (PDE1-PDE5) and on porcine coronary arteries vasorelaxation.

Materials and Methods

Plant extract

Stem barks of *A. leiocarpus* were collected in May 2006 in the area of Loumbila (zone of savanna), locality located at 20 km in the East of Ouagadougou (Burkina Faso). Voucher specimen (N°1544) was deposited at the herbarium of the Department of Forest Production, National Centre for Scientific and Technological Research, Ouagadougou Burkina Faso. The collected sample was air-dried deprived of solar light, dust and was powdered. The extract of *ALF* was prepared starting from the powder of the plant as follows: stem barks of *A. leiocarpus* were crushed and then an aqueous decoction was carried out. Exhaustion by the dichloromethane followed by dry evaporation (35°C) led to the extract of *ALF* used for the pharmacological investigations. The extract was prepared freshly in DMSO 100% then diluted in water in order to obtain a final concentration in the tank lower or equal to 0.02% (this concentration not having a notable effect on the activity of relieving, (Lugnier 2006).

Cyclic nucleotide phosphodiesterase study

PDE1, PDE3, PDE4 and PDE5 were isolated by anion exchange chromatography from bovine aortic smooth muscle cytosolic fraction according to Lugnier et al. (1986). PDE2 was isolated from human platelets following the method indicated in Kameni et al. (2001). Purified PDEs were stored until use at -80°C in small aliquots (200 µL). PDE activities were measured by radioenzymatic assay as previously described in detail (Thaseldar,) at a substrate concentration of 1 µM cAMP or 1 µM cGMP in the presence of 10,000 cpm [³H]-cAMP or [³H]-cGMP as tracers. The buffer solution was of the following composition: 50 mM Tris-HCl (pH 7.5), 2 mM magnesium acetate, 50 mg BSA. PDE1 was assayed at 1 µM cGMP in basal state (1 mM EGTA) or in calmodulin activated state (18 nM calmodulin with 10 µM CaCl₂). PDE2 was evaluated at 1 µM cAMP + 1 mM EGTA in basal state (without 5 µM cGMP) and in activated state (in presence of 5 µM cGMP). PDE3 and PDE4 were assayed at 1 µM cAMP + 1 mM EGTA. To prevent the influence of reciprocal cross-contamination between PDE3 and PDE4, the studies were always carried out in the presence of 50 µM rolipram (a generous gift of Schering, Berlin, Germany) for PDE3 and in presence of 50 µM cGMP for PDE4. PDE5 activity was measured at 1 µM cGMP in the presence of 1 mM of EGTA. The concentration of compounds that produced 50% inhibition of substrate hydrolysis (IC₅₀) was calculated by non-linear regression analysis (GraphPad Prism software, San Diego, Ca) from concentration-response curves including 6 different concentrations of inhibitors. The results were expressed as Means ± standard error of the mean (SEM) of at least three determinations in duplicate.

Vascular reactivity studies

Left anterior descending porcine coronary arteries (obtained from the local slaughterhouse) of either sex were cleaned of connective tissue and cut into rings (3–4 mm in length). As indicated the endothelium was removed by rubbing the intimal surface of rings with a pair of forceps. Rings were suspended in organ baths containing oxygenated (95% O₂ and 5% CO₂) Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.18, CaCl₂ 1.25, NaHCO₃ 25, and D-glucose 11, pH 7.4, 37 °C) and the cyclooxygenase inhibitor indomethacin (10 µM), for the determination of changes in isometric tension. Following equilibration for 90 min under a resting tension of 5 g, rings were contracted with KCl (80 mM). After a 30 min washout period, rings were contracted with the thromboxane mimetic U46619 (1-3.10⁻⁸ M) to about 80% of the maximal contraction before addition of bradykinin (3.10⁻⁷ M) to check the presence of a functional endothelium. Rings were considered with and without endothelium when the relaxation to bradykinin is superior or equal to 80% and inferior or equal to 0% respectively. After washout and a 30 min equilibration period, rings were again contracted with U46619 before construction of a concentration–relaxation curve to *ALF*. In some experiments, rings were exposed to an inhibitor for 30-45 min before the addition of U46619.

Drugs and chemicals

L-nitro-arginine (L-NA), 4-aminopyridine (4-AP), Charybdotoxine (CTX), Apanin (APA), Baryum (Ba²⁺), Ouabain, 1 H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide dihydrochloride (H89) were all obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). U46619 (9,11-dideoxy-11 α , 9 α -epoxymethanoprostaglandin F₂ α) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Data and statistical analysis

Rings from the same heart were used for one treatment only. Results are expressed as the Mean ± S.E.M. and *n* represents the number of rings used in the experiments. Relaxation was expressed as percentages of the U46619-induced contraction. IC₅₀ and EC₅₀ values were determined using a curve-fitting program (GraphPad prism 5.00.288). Two way analysis of variance (ANOVA) and post-hoc Bonferroni's test were used to determine the significant differences between treatments. A *P*-value less than 0.05 was considered as statistically significant.

Results

Effects of ALF extract on purified PDE1 to PDE5

In vascular smooth muscle, cGMP degradation is mainly due to PDE1 which is activable by the calcium calmodulin complex, and by PDE5 which specifically hydrolyses cGMP (Keravis et al., 1980, Lugnier et al 1986, Komasa et al., 1991), whereas cAMP degradation is due to PDE3 and PDE4 (Lugnier et al., 1986, Komasa et al., 1991, Orallo et al 2004). Endothelial cells, in opposite of smooth muscle cells does not contain PDE1 but PDE2 which hydrolyses both cAMP and cGMP and is activable by cGMP (Lugnier et Schini, 1990, Keravis et al., 2007).

The dose-response of PDEs inhibition (Figure 1A-B) and Table I reporting the IC₅₀ values of ALF for PDE1 to PDE5 show that ALF inhibited potently and preferentially cGMP degradation by PDE1 with an IC₅₀ value of 1.6 ± 0.6 µg/mL. In the same range (IC₅₀=2.8 ±0.2 µg/ml) ALF inhibited PDE2, a cGMP-activated enzyme that is only present in endothelial cells. PDE5, was also potently inhibited by ALF (IC₅₀=7.6±3.5 µg/ml). The potencies of ALF on cAMP hydrolyzing isoenzymes was lesser, being more effective on PDE4 (IC₅₀= 17.6±3.5 µg/ml) than on PDE3 (60.9 ± 1.8 µg/ml). These data clearly show that ALF preferentially inhibits the way of degradation of cGMP than those of cAMP.

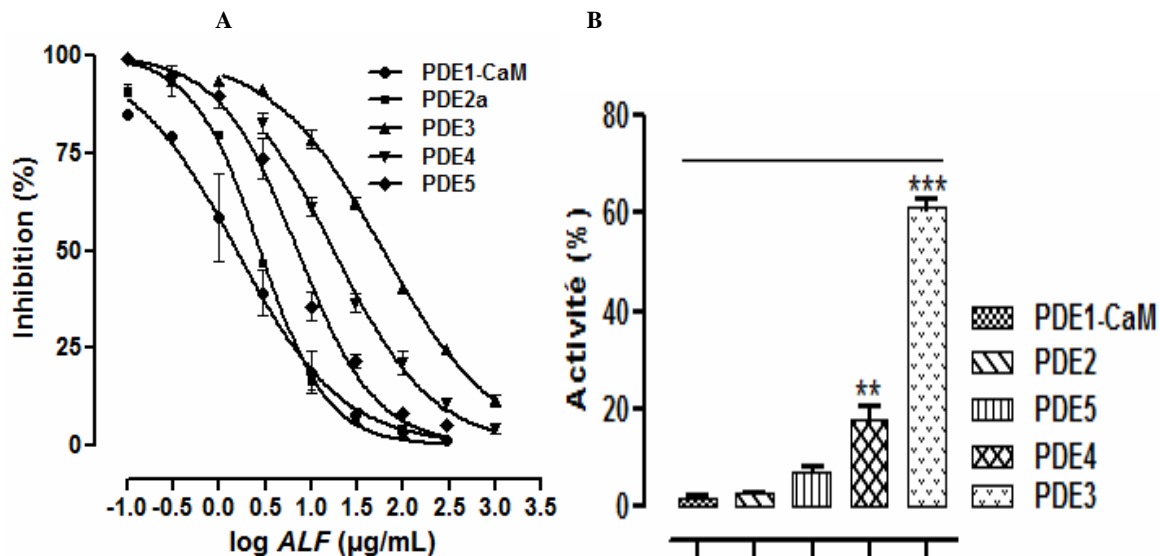


Figure 1: Inhibition induced by cumulative concentrations of ALF on PDEs. (n= 3 duplicate different experiments, *p<0.05 as compared with PDE 1, ANOVA follow by Bonferroni's test ; (A). Histogram showed the IC₅₀ statistical results of ALF activity on PDEs (B). (n = 3 different experiments, p<0.05 versus PDE 1, ANOVA with post-hoc Bonferroni's test was use).

Table I: Effect of ALF on purified vascular PDE1-PDE5 (IC₅₀, µg/ml)

isoenzyme	PDE1	PDE2	PDE3	PDE4	PDE5
³ H Substrate	cGMP	cAMP	cAMP	cAMP	cGMP
Modulator	CaM/Ca ²⁺	+ 5 µM cGMP	-	-	-
IC ₅₀ (µg/mL)	1.6 ± 0.6	2.8 ± 0.2	60.9 ± 1.8	17.6 ± 3.5	7.1 ± 1.9

IC₅₀ is defined as the concentration of the inhibitor (in µg/mL) required to reduce the cyclic nucleotide hydrolyzing activity of tested PDEs by 50%. Data represent the mean±s.e.m. of 3 independent experiments

Table II: Effects of ALF on porcine coronary artery ((EC₅₀, µg/ml)).

	With endothelium (E)	E+L-NA	E+CTX+APA	E+L-NA+CTX+APA	Without endothelium
CE ₅₀ (µg/mL)	2.3±1.0	17.2 ± 2.3-	2.1 ± 1.0	18.8 ± 1.9	11.5 ± 2.0

ALF induced endothelium-dependant and endothelium-independent relaxation on porcine coronary artery

The endothelium-dependent vasorelaxant effects of ALF were examined on isolated porcine coronary artery rings with and without endothelium. Cumulative concentrations of ALF (0.1–100 µg/mL) produced concentration-dependent vasorelaxations both in endothelium-intact and endothelium-denuded artery rings. However, ALF induced a significant shift to the left in the response curve after endothelium denudation with significant increase in CE₅₀ (from 2.3±1.0 to 11.5±2.0 µg/ml), revealing that ALF-induced both endothelium-dependent and endothelium-independent vasodilatations (Figure 2, Tableau II).

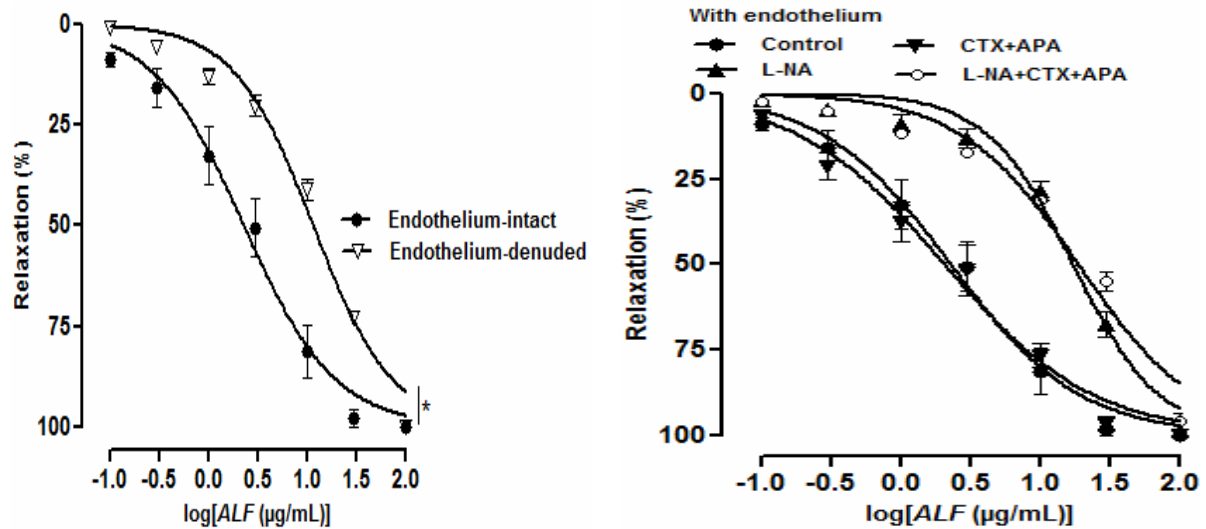


Figure 2 Left panel: Vasorelaxations induced by cumulative concentrations of ALF in endothelium-intact and endothelium-denuded porcine coronary rings pre-contracted with U46619. ALF induced relaxant responses were significantly different in endothelium-intact and endothelium-denuded coronary rings at the indicated ALF concentrations. (n= 3 to 4 different experiments, *p<0.05). ANOVA follow by Bonferroni's test. **Figure 3 Right Panel:** Concentration-responses curves of ALF induced relaxations in porcine coronary artery rings. Intact endothelium rings were contracted with U46619 before the addition of increasing concentrations of ALF. Rings with endothelium were also incubated with either N^o-nitro-L-arginine (L-NA, 300 µM, an inhibitor of eNOS), charybdotoxin (CTX, 100 nM) plus apamin (APA, 100 nM; two inhibitors of EDHF-mediated relaxations) or the combination of L-NA plus CTX and APA for 30 min before addition of U46619. Experiments were performed in the presence of indomethacin. (n=3 to 4 different experiments. p< 0.05 versus control with endothelium was considered as significant, ANOVA follow by Bonferroni's test).

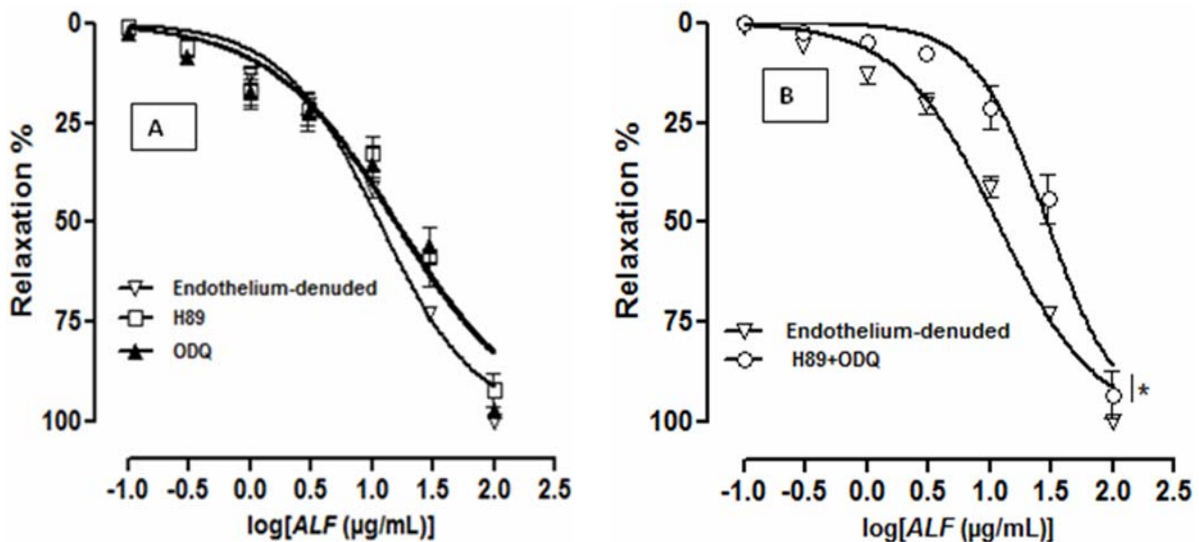


Figure 4: Concentration–response effect of ALF in U46619 pre-contracted endothelium-denuded coronary artery rings , or in the presence of N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide dihydrochloride (H89, 100nM) and 1H-[1,2,4]-Oxadiazole-[4,3-a]-quinoxalin-1-one (ODQ, 10 µM) (A), or in the presence of the combination of H89 plus ODQ (B).(n= 3, *p<0.05 versus endothelium-denuded, ANOVA follow post-hoc Bonferroni's test)

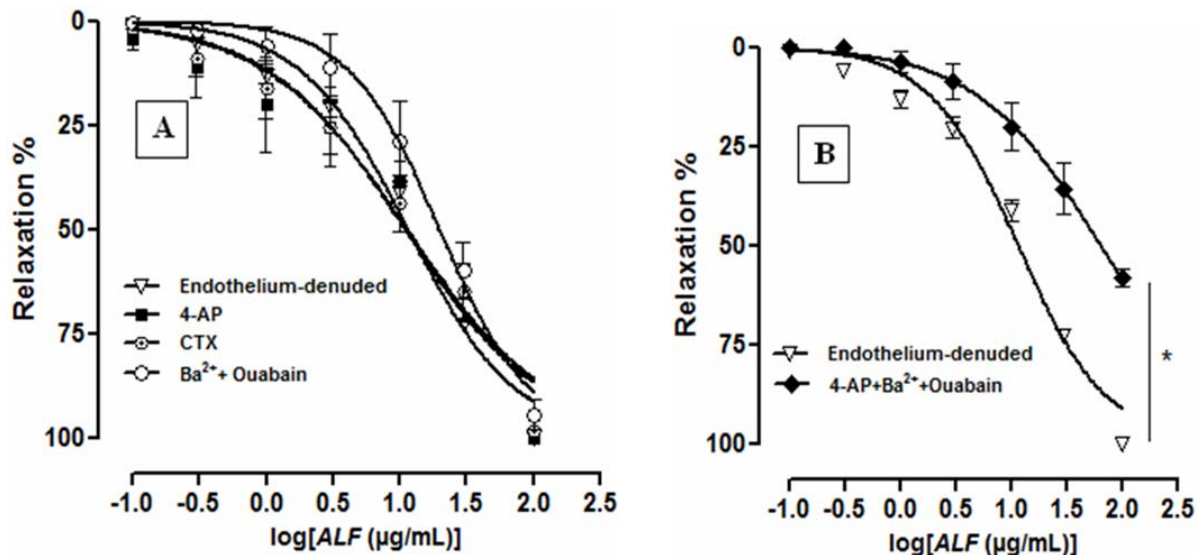


Figure 5: Without endothelium, rings were pre-incubated with 4-aminopyridine (4-AP, 1 mM), CTX (10µM) or Ba²⁺ (30µM) + ouabain (3 µM) (A) and with the association of 4-AP plus Ba²⁺ plus ouabain (B). All experiments were performed by the presence of indomethacin (10µM). (n=3 to 4 different experiments, *p<0.05 versus endothelium-denuded, ANOVA follow by post-hoc Bonferroni's test).

Effects of NO and EDHF

In endothelium-intact coronary rings, pretreatment with EDHF inhibitors (CTX plus APA) did not affect relaxation to ALF. But, pretreatment with a nitric oxide synthase (NOS) inhibitor L-NA or with both of them (L-NA plus CTX plus APA), significantly reduced the vasorelaxing effect of ALF (Figure 3). Therefore, the EC₅₀ values (Table II) were in the same range with the EC₅₀ value obtained in absence of endothelium, strengthening the endothelium dependent vasorelaxing effect of ALF.

Modulation of ALF effects by PKA inhibitor and soluble Guanylyl cyclase inhibitor.

In endothelium-denuded rings, this relaxant effect of ALF on the coronary tissues was not affected by the presence of H89 or ODQ alone (Figure 4 A). However, in the presence of H89 plus ODQ, the relaxation curve of ALF was slightly but significantly shifted suggesting a probable synergetic action of PKA and soluble guanylyl cyclase responsible to ALF relaxation (Figure 4B).

Effects of potassium channel antagonists on ALF-induced of pig coronary arteries relaxation.

ALF relaxation in endothelium-denuded rings was not affected by 4-aminopyridine (4-AP, a voltage-sensitive potassium-channel inhibitor) or charybdotoxin (CTX, an IK_{Ca} inhibitor) nor baryum (Ba²⁺) plus ouabain (an IK₁ and Na⁺/K⁺-ATPase inhibitors) (Figure 5 A). However, the addition of 4-AP plus Ba²⁺ plus ouabain led to a reduction of ALF maximal effect, in the order of 60% (Figure 5B).

Discussion

In this study, we demonstrated that the vasorelaxant effects of ALF is mediated via NO/sGC/cGMP and cAMP pathways, potassium (K⁺) and Na⁺/K⁺-ATPase channels opening. There are clear evidences from previous studies that NO/cGMP and cAMP pathways regulate vascular tone (Komas al. 1991; Lugnier and Komas 1993). Nitric oxide (NO) is an important regulator vascular function. The direct effect of NO on vascular tone is brought about by cGMP formation in vascular smooth muscles cells. Several NO-related compounds, such as sodium nitroprusside and endogenous NO activate soluble guanylate cylase (sGC), elevate cGMP and relax smooth muscles (Katsuki et al., 1977).

The present findings indicate that ALF is a powerful endothelium-dependent vasodilator of porcine coronary arteries. Relaxations to ALF in endothelium-intact rings were significantly reduced by L-NA without affecting the maximal relaxation indicating an important role for NO. The inhibition of EDHF-mediated responses with charybdotoxin plus apamin did not affect relaxations to ALF. In contrast to red wine extract (Ndiaye et al. 2005), the relaxation to ALF persisted in the presence of the combination L-NA, charybdotoxin and apamin on pig coronary artery. The persistent relaxation to ALF was not due to the formation of relaxing prostanoids; since all experiments were performed in the presence of indomethacin. The relaxation induced by ALF was significantly affected by disruption of the endothelial layer (5 fold increase in EC₅₀), without being suppressed, suggesting that the site of action of ALF is in the vascular smooth muscle. Interestingly, ALF in the same

range of concentration inhibit PDE1 and PDE2, suggesting that in presence of endothelium both PDE1 and PDE2 were inhibited by *ALF*.

Indeed, relaxation to *ALF* was not affected by the presence of a PKA inhibitor (H89) and an inhibitor of soluble guanylyl cyclase (ODQ) but their combination significantly reduced *ALF* relaxation in endothelium denuded artery suggesting a probable synergetic action of both of them. The relaxing effect of *ALF* may be also related to its ability to inhibit the activity of cAMP-phosphodiesterase (Ferrell et al., 1979). Vascular PDEs are differentially distributed in endothelial cells and vascular smooth muscle cells, and there is evidence of interaction between cGMP and cAMP degrading enzymes both in vascular endothelial and smooth muscle cells.

The results showed that PDE1, 2, 4 and 5 were inhibited by *ALF* with IC_{50} = 1.55 ± 0.56 , 2.79 ± 0.23 , 17.61 ± 3.50 and 7.11 ± 1.90 μ g/mL respectively. These results suggested that *ALF* may block the way of cGMP and cAMP degradation and then increase cAMP and cGMP level (Lugnier et al. 1999). These cyclic nucleotide accumulations may explain the relaxation mechanism of *ALF* on endothelium-intact and endothelium-denuded coronary artery rings. Our preliminary phytochemical screening identified some chemical compounds from *ALF* extract such as glycosides (sterols and triterpen) and alcaloides (Mann et al., 2009) which show a large variety of pharmacological action (Kim et al. 1999). It's reported that some glycosides could inhibit cyclic nucleotide phosphodiesterase (Orallo et al. 2004). Hence, taken in conjunction with these results, *ALF* may activate the NO/sGC/cGMP and cAMP pathway and inhibit the PDEs activity, and therefore could elevate the intracellular cGMP and cAMP levels leading to vascular smooth muscle relaxation.

On PDE3, the IC_{50} = 60.85 ± 1.76 μ g/mL of *ALF* was lower than the other PDEs inhibited but this result raised lightly significant inhibition. Several studies showed that PDE3 is present in vascular smooth muscle and is inhibited by cGMP and then increases cAMP (Beavo 1995; Lugnier et al. 1999; Mercapide et al. 1999; Sandner et al. 1999). Indeed, the nonspecific PDE inhibition activity may increase cAMP and cause relaxation in smooth muscles (Fischer et al. 1992). Moreover, a major focus of studies in vascular biology has been the role of the endothelium in modulating tone of vascular smooth muscle by the release of NO, stimulation of sGC, and the subsequent increase in intracellular cGMP (Lamping 2001). Although most of these studies suggest that vasorelaxation to many agents is mediated by the release of cGMP or cAMP alone, several of them have suggested that some "typical endothelium-independent" vasodilators may also release NO and activate sGC (Wang et al. 1993; Cardillo et al. 1997; Dawes et al. 1997). Thus, *ALF* relaxation in endothelium-denuded artery involves PDEs inhibition on vascular smooth muscle cells.

To determine whether K^+ channels mediated endothelium-independent relaxation of coronary rings by *ALF*, we used different potassium channel inhibitors. Pretreatment with 4-AP and CTX used only or Barium plus ouabain did not affect *ALF*-induced relaxation. But this relaxation effect was markedly affected by the association of 4-AP plus Ba^{2+} plus ouabain and reduced maximal relaxation to about 60%. We suggested that *ALF*-induced vasorelaxation might be partly via the opening of voltage-dependent K^+ channel (K_v), IK_1 and Na^+/K^+ -ATPase channels.

In summary, these findings suggest that *ALF* activity on porcine coronary artery is endothelium-dependent and endothelium-independent. This is mostly on one hand, mediated via NO/sGC/cGMP pathway, stimulation of the K^+ , Na^+/K^+ -ATPase channels, on the other hand via inhibition of PDE activity and might contribute to explain the pharmacological effect of *ALF*.

Acknowledgements

We would like to thank the Coopération et action Culturelle, Fond de Solidarité Prioritaire, Ambassade de France au Burkina Faso for their financial assistance. This work was also supported by grant to Belemnaba Lazare from Service de Coopération et d'Action Culturelle de l'Ambassade de France. We would also like to thank International Foundation for Sciences (IFS) for support.

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