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## **Cognac Polyphenolic Compounds increase Bradykinin-induced Nitric Oxide production in Endothelial Cells**

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**Short title** : Synergistic effect of Polyphenols and Bradykinin on NO production

## Summary

We recently reported that in vitro, Cognac polyphenolic compounds (CPC) induce NO-dependent vasorelaxant effects and stimulate cardiac function. In the present study, we aim to investigate the effect of CPC on both nitric oxide (NO) and superoxide anions ( $O_2^-$ ) production in cultured human endothelial cells. Beside, its effect on the capacity of bradykinin (BK) to produce NO was also tested. The role and sources of  $O_2^-$  in the concomitant effect of BK plus CPC were pharmacologically determined. NO and  $O_2^-$  productions were measured using electron paramagnetic resonance technique using specific spin trappings. Both, CPC and BK induced an increase in NO production in human endothelial cells. The combination of both further enhanced NO release. The capacity of CPC plus BK in increasing NO signal was blunted by the NO synthase inhibitor,  $N^G$ -nitro-L-arginine methyl ester, and was enhanced in the presence either of superoxide dismutase or catalase. Beside, CPC plus BK response was greater after inhibition either of NADPH oxidase by apocynin or xanthine oxidase by allopurinol but was not affected by rotenone. CPC did not affect  $O_2^-$  production neither alone nor after its increase upon lipopolysaccharide treatment. Finally, the capacity of BK alone in increasing NO was enhanced either by apocynin or allopurinol. Altogether, these data demonstrate that CPC is able to directly increase NO production without affecting  $O_2^-$  and enhances the capacity of BK to produce NO in human endothelial cells. The data highlight the ability of BK to stimulate not only NADPH oxidase but also xanthine oxidase-inhibitor sensitive mechanisms that reduce its efficiency in increasing NO either alone or in the presence of CPC. These results bring pharmacological evidence of vasculo-protection by CPC via its potentiating effect of BK response in terms of endothelial NO release.

**Keywords :** Polyphenols ; Bradykinin ; Reactive oxygen species ; Endothelial cells

## Introduction

Endothelium-derived nitric oxide is a pivotal mediator involved in a variety of biological process, including regulation of smooth muscle tone and endothelium-dependant relaxation, platelet activation and aggregation, neurotransmission and vascular cells signalling (Randriamboavonjy and Fleming 2005; Busse and Fleming 2006; Moncada and Bolanos 2006; Moncada and Higgs 2006; Villalobo 2006). The endothelial formation of NO can be increased both by pharmacological and physiological agonists (e.g. bradykinin) and this effect is dependent on the interaction of calcium/calmodulin with endothelial NO synthase (eNOS) (Boulanger *et al.* 1990; Schini-Kerth 1999; Bae *et al.* 2003; Schneider *et al.* 2003), the PKA-dependent phosphorylation of eNOS (Bae *et al.* 2003). Interesting in the mechanism by which polyphenols can produce NO in endothelial cells, despite the involvement of several mechanisms, such as an increase in intracellular calcium and the activation in tyrosine kinases (Stoclet *et al.* 1999; Martin *et al.* 2002), up-regulation of mRNA expression eNOS (Nie *et al.* 2006), increasing evidence suggests additional roles for reactive oxygen species (ROS) (Andriantsitohaina 1999; Jaimes *et al.* 2001; Ndiaye *et al.* 2005). However, the interpretation of these studies investigating vascular NO production by physiological or pharmacological agonists or plant polyphenols need to be considered together rather than in isolation. With regard to plant polyphenols, Cognac belongs to alcoholic drinks rich in polyphenols and moderate consumption may have potential beneficial effects on health. We recently reported that *in vitro* oral treatment with Cognac polyphenolic compounds (CPC) appears to have no impact *in vivo* on blood pressure, heart rate of the rats or on cardiac contractility *ex vivo*; however, it reduces heart work, decreases both the infarct size after an ischaemia–reperfusion (Ralay Ranaivo *et al.* 2004). Accordingly, we sought to evaluate the interactions and relationships between bradykinin, a physiologically active peptide and potent endothelium-dependent vasodilator, and CPC on the production of NO in human endothelial cells.

## Methods

### *Cell culture*

EAhy 926 cells, a human aortic endothelial cell hybridoma, were cultured in growth medium (DMEM, Ham's F-12, 1:1) supplemented with 1% l-glutamine, 1% HAT, 1% NEAA, 1% Na-pyruvate, 1% streptomycin/ penicillin (Cambrex, Verviers, Belgium), and 10% FBS (Invitrogen, Cergy Pontoise, France). All experiments were performed in confluent Eahy cells from passages 8 to 12 and serum-deprived for at least 24 h.

For the measurement of NO production, the culture medium was removed and replaced by fresh Hank's Balanced Salt Solution (HBSS), pH 7.4, containing 1M N-2-hydroxyethylpiperazine-N9-2-ethane-sulfonic acid (HEPES), 1M L-Arginine, 1M CaCl<sub>2</sub> and 0.1% BSA. After 60 min, cells were treated with bradykinin (BK, 40 μM, Sigma-Aldrich, Saint Quentin, Fallavier, France), alone or 15 min prior treatment of CPC (0.03 mg/ml in 0.5% glucose) for 30 min. CPC was used at a concentration at which it produced maximal relaxation on aortic rings with functional endothelium (Ralay Ranaivo *et al.* 2004).

Parallel sets of experiments were performed in the presence of either superoxide dismutase, (SOD, 100 UI/ml), catalase (100 UI/ml), the xanthine oxidase inhibitor, allopurinol (50 μM) (Sigma-Aldrich), the potent NADPH oxidase inhibitor, apocynin (100 μM, Sigma-Aldrich), the mitochondrial complex I inhibitor, rotenone (5 μM, Sigma-Aldrich) or the NOS inhibitor, nitro-L-arginine (L-NAME, 100 μM, Sigma-Aldrich).

For the measurement of superoxide anions (O<sub>2</sub><sup>-</sup>), cells were treated with lipopolysaccharide (LPS, 10μg/ml, Sigma-Aldrich) for 3h and then with or without CPC (0.03 mg/ml in 0.5% glucose) for further 60 min.

### **NO spin trapping and electronic paramagnetic resonance (EPR) studies**

Detection of NO production was performed using the technique with Fe<sup>2+</sup> diethyldithiocarbamate (DETC, Sigma-Aldrich) as spin trap. Briefly, after 30 min of CPC treatment in absence or presence of different inhibitors, the medium was replaced with 250 μL of Krebs Hepes Buffer (KHB), and then treated with 250 μL of colloid Fe(DETC)<sub>2</sub> and

incubated for 45 min at 37 °C. NO detection was measured *in situ* by EPR. Values are expressed in units/ $\mu\text{g}/\mu\text{L}$  of endothelial cell proteins.

NO measurement was performed on a table-top x-band spectrometer Miniscope (Magnettech, MS200, Berlin, Germany). Recordings were made at 77 °K, using a Dewar flask. Instrument settings were 10 mW of microwave power, 1 mT of amplitude modulation, 100 kHz of modulation frequency, 150 s of Sweep time and 5 scans. Signals were quantified by measuring the total amplitude, after the correction of baseline as previously described [15]. The quantitative measurement of the NO-Fe(DETC)<sub>2</sub> signal amplitude was reported to the relative units for protein concentration of cells.

#### ***Determination of superoxide anion generation***

For the detection of superoxide anion production, EaHy 926 cells were incubated for 45 min at 37°C in KHB (pH 7.4) containing 5  $\mu\text{M}$  DETC, 25  $\mu\text{M}$  deferoxamine and 500  $\mu\text{M}$  1 hydroxy- 3 methoxycarbonyl 2,2,5,5- tetramethylpyrrolidin (CMH, Noxygen, Germany). Briefly, deferoxamin and DETC were dissolved under argon gas bubbling in ice-cold KHB. They were rapidly mixed to the CMH solution, which was used immediately. Then, cells were detached with trypsin-EDTA (Invitrogen, Cergy Pontoise, France) and frozen in plastic tubes to form rods. Electron spin resonance (ESR) spectra were recorded on a table-top x-band spectrometer Miniscope (Magnettech). The ESR instrument settings were as follows : BO-field, 3327G ; microwave power, 1mW (20 db) ; modulation amplitude, 5 G; sweep time, 60s ; field sweep, 60G (BO-sweep) ; gain 500 (5x2). Signals were quantified by measuring the total amplitude, after correction of baseline and normalisation to the protein concentration of cells.

#### ***CPC contents***

The enriched and lyophilized CPC powder was provided by the 'Bureau Interprofessionnel du Cognac' and contains, as previously described (Ralay Ranaivo *et al.*, 2004) in mg/g of powder : gallic acid, 2.28 ; ellagic acid, 15.17 ; 5-hydroxy-methylfurfural, 0.17 ; syringic acid, 0.74 ; vanillin, 0.45 ; syringaldehyde, 2.02, scopoletin, 0.03 ; coniferaldehyde, 2.35 ;

sinapaldehyde, 8.21. The total polyphenols content was about 48.8% determined by colorimetric Follin-Ciocalteau method using gallic acid as standard.

### ***Statistical analysis***

All values are mean  $\pm$  S.E.M.  $n$  represents the number of experiments as appropriate. Statistical analysis was carried out using one-way ANOVA. A Fisher's PLSD test was used for Post Hoc comparison.  $P < 0.05$  was considered to be statistically significant.

## **Results**

### ***CPC potentiates BK-induced NO production.***

As illustrated on figure 1, exposure of EAhy 926 endothelial cells with BK led to an expected increase in NO production ( $+ 32 \pm 6.8\%$  ;  $P < 0.05$ ). CPC also significantly enhanced NO production ( $+ 21 \pm 10.5\%$   $P < 0.05$ ), and this to the same extent to that triggered by BK. Co-incubation of CPC with BK induced further increase in NO release ( $+ 82 \pm 8\%$  ,  $P < 0.01$ ). The capacity of CPC plus BK in increasing NO signal was blunted by the NOS inhibitor, L-NAME ( $- 22 \pm 7.7\%$  ,  $P < 0.05$ ).

### ***The additive effects of CPC and BK on NO production was blunted by the production of ROS sensitive to both NADPH oxidase- and xanthine-oxidase- inhibitors.***

As shown on figure 2A, SOD, which catalyses the reduction of  $O_2^-$  to hydrogen peroxide ( $H_2O_2$ ), significantly enhanced NO production induced by BK plus CPC ( $+ 41 \pm 8\%$  ,  $P < 0.01$ ). Moreover, catalase, which is known as an efficient enzyme for the conversion of  $H_2O_2$  into water and oxygen, significantly increased NO production elicited by BK plus CPC ( $+ 21 \pm 3.4\%$  ,  $P < 0.05$ ). Altogether, these data strongly suggest the involvement of ROS in response to either BK and/or CPC that blunted the production of NO.

The sources of endogenous ROS implicate was then further investigated by testing the effect of different pharmacological inhibitors (figure 2B). Blockade either of NADPH oxydase by apocynin ( $+ 38 \pm 15.7\%$  ,  $P < 0.05$  ) or the cellular xanthine oxidase by allopurinol ( $+ 64 \pm 25.7\%$  ,  $P < 0.01$ ) significantly enhanced NO production elicited by CPC plus BK. In contrast,

the inhibitor mitochondrial respiratory chain at site I, rotenone, did not affect the response to both agents.

***Superoxide anions production originate from BK but not from CPC stimulation of endothelial cells.***

To test which compound used was able to promote  $O_2^-$  production that blunted the increase of NO production elicited by addition of BK plus CPC, we directly measured either the capacity of CPC to increase  $O_2^-$  or the effect of pharmacological inhibitors on BK-induced NO response in endothelial cells.

For the former (figure 3A), CPC alone was not able to significantly increase  $O_2^-$  production under the experimental conditions used. This was not linked to the incapacity of EAhy to produce  $O_2^-$  inasmuch treatment of these endothelial cells with LPS resulted an increase of  $O_2^-$  generation ( $+ 47 \pm 23.4 \% ; P < 0.05$ ). Moreover, the ability of LPS to increase  $O_2^-$  was not significantly enhanced by concomitant treatment of the cells with CPC ( $+ 67 \pm 6.5\%$ ). The increase in  $O_2^-$  was abolished either by the superoxide mimetic MnTMPyP or by the cell-membrane permeable radical scavenger, tempol (data not shown).

Interestingly, the capacity of BK in increasing NO production was significantly increased in the presence of either apocynin ( $+ 83 \pm 38.9 \% , P < 0.05$ ) or allopurinol ( $+ 107 \pm 45.1 \% , P < 0.05$ ; figure 3B).

Altogether, these data demonstrate that BK was able to stimulate NADPH oxidase- and xanthine oxidase-inhibitors sensitive mechanisms that reduced its efficiency in increasing NO either alone or in the presence of CPC. They also highlight the fact that CPC enhanced NO production without an effect of ROS production.

## **Discussion**

This present study provides new findings into the interaction between BK, a physiologically active peptide of the kinin group, and polyphenols from a non-alcoholic extract of cognac. In human endothelial cells line, NO release is significantly modulated by interaction of BK and

CPC, following activation of NO synthase and enzymes that generate intracellular  $O_2^-$ . Indeed, the data demonstrate that CPC is able to directly increase NO production without affecting  $O_2^-$  and enhances the capacity of BK to produce NO in human endothelial cells. The data highlight the ability of BK to stimulate not only NADPH oxidase- but also xanthine oxidase-inhibitor sensitive mechanisms that reduce its efficiency in increasing NO either alone or in the presence of CPC.

The mechanism by which BK and others endogenous vasoactive substances such as acetylcholine, adenosine and arginine, stimulate NO production in vascular tissues has been extensively described. These include a transient phosphorylation of the  $B_2$  receptor on tyrosine residues in cultured endothelial cells (Marrero *et al.* 1999), a calcium-store-dependent calcium influx as well as a PI3-kinase activation (Kitayama *et al.* 2000; Schneider *et al.* 2003) (Tran *et al.* 2000) and a protein kinase A-dependent phosphorylation of eNOS-Ser(1179) (Bae *et al.* 2003).

Of particular interest is the role of ROS in BK-induced NO release. In bradykinin-stimulated coronary arteries, antisense oligonucleotides against CYP 2C (a cytochrome P450 isozyme homologous) potentiated NO-mediated relaxation (Fleming *et al.* 2001). Folates,  $O_2^-$ , and peroxynitrite scavengers restore the NO-generating activity to eNOS, collectively suggesting that extracellular cellular redox state plays an important role in NO-generating function of this enzyme (Zhang *et al.* 2000). In porcine aortic endothelial cells, Jaimes *et al.* have reported that  $H_2O_2$  impairs NO production in response to both receptor-dependent and receptor-independent agonists and that these effects are due, at least in part, to inactivation of eNOS cofactors, whereas HOCl inhibits NO production by interfering with receptor-operated mechanisms at the level of the cell membrane (Jaimes *et al.* 2001). Furthermore, endogenous  $H_2O_2$  derived from NAD(P)H oxidase, mediates endothelial NO production in response to another agonist such as angiotensin II in bovine aortic endothelial cells (Cai *et al.* 2002). In the present study, we highlight in human endothelial cells that the capacity of BK in increasing NO production was blunted by its ability to activate both NADPH oxidase- and

xanthine oxidase-inhibitor sensitive mechanisms. Although, the two enzymes are known to enhance  $O_2^-$  production in endothelial cells, especially for angiotensin II stimulation of NADPH oxidase. We report the first time the capacity of BK to activate not only NADPH but also xanthine oxidase. It might be possible that  $O_2^-$ -induced changes in the disturbance of cytosolic calcium homeostasis participate on the blunting effect of either NADPH or xanthine oxidase inhibitor on NO production elicited by BK. However, it is likely that  $O_2^-$  produced by such enzymes interacts with NO to produce peroxynitrite and thus reduced NO release upon BK treatment.

With regard to CPC, we reported that CPC induce a vasorelaxant response of rat aortic rings in the presence but not in the absence of functional endothelium suggesting the obligatory role of the endothelium (Ralay Ranaivo *et al.* 2004). The involvement of prostacyclin was unlikely as the vasorelaxant response to CPC was not altered in the presence of the cyclo-oxygenase inhibitor, indomethacin. However, the relaxant effect of CPC was totally abolished in the presence of the NO synthase inhibitor, L-NAME. These data suggest that endothelial relaxation of rat aorta by CPC involved NO-dependent pathway. The implication of NO could result from an increased production of NO or from a diminution of its breakdown. In the present study, we demonstrate that CPC was able to directly stimulate NO production from endothelial cells without affecting  $O_2^-$  production. Thus, the ability of CPC to enhance endothelial NO production was not linked to its anti-oxidant properties. These results also contrast with our data with red wine polyphenolic compounds (RWPC) showing the involvement of  $O_2^-$  in the cytosolic calcium increase evoked by these compounds leading to the activation of enzymes involved in the release of endothelial relaxant factors including eNOS (Martin *et al.* 2002; Duarte *et al.* 2004). Moreover, our results are not in accordance with the study showing that RWPC promotes the release of endothelial NO through the redox sensitive PI3/Akt pathway (Ndiaye *et al.* 2005). It is likely that the nature of polyphenols involved in the response to CPC and RWPC might explain the differential mechanisms of these two compounds to promote NO release. For RWPC, the molecular identity of

polyphenols responsible for this effect may include oligomeric condensed tannins and anthocyanins (Andriambelosen *et al.* 1998). Among the identified components of CPC, phenolic acids (gallic acid and vanillic acid) are unlikely to be implicated because they fail to induce relaxant response in aorta. We cannot exclude that ellagic acid might participate in the endothelial-dependent relaxation of CPC by potentiating the action of other compounds through its antioxidant capacity. Finally, we could not exclude that unidentified compounds, present in very low proportion in CPC, mediated the capacity of CPC to increase endothelial NO production.

Finally and most importantly, we thought that vascular NO production by physiological or pharmacological agonists, or plant polyphenols need to be considered together rather than in isolation. Here we demonstrate that BK and CPC acted synergistically to enhance endothelial NO production. Beside, both compounds probably mediate their effect via distinct pathways at least with regard to activation of enzymes that promote  $O_2^-$  production such as NADPH oxidase and xanthine oxidase. Although, the mechanism by which BK promote NO release within endothelial cells is well described (see above), that of CPC requires further investigations. Nevertheless, we report here that the potentiating effect of CPC on BK-induced NO release probably plays a role in the vasculo-protection induced by compounds in Cognac.

In conclusion, the present study shed light on the synergistic effect of CPC and BK in inducing NO production in human endothelial cells. The data underscore the ability of BK to stimulate not only NADPH oxidase- but also xanthine oxidase-inhibitor sensitive mechanisms that reduce its efficiency in increasing NO either alone or in the presence of CPC as well.

## Figures Legendes

Fig. 1

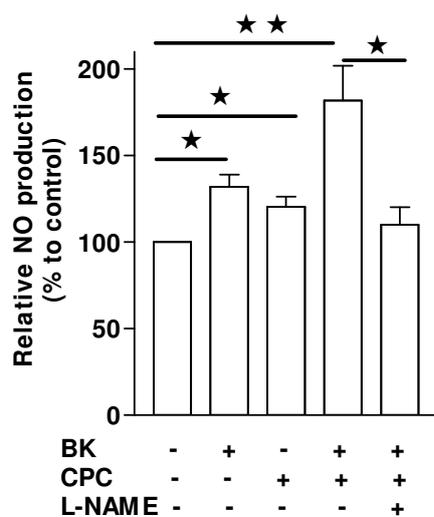


Fig. 2

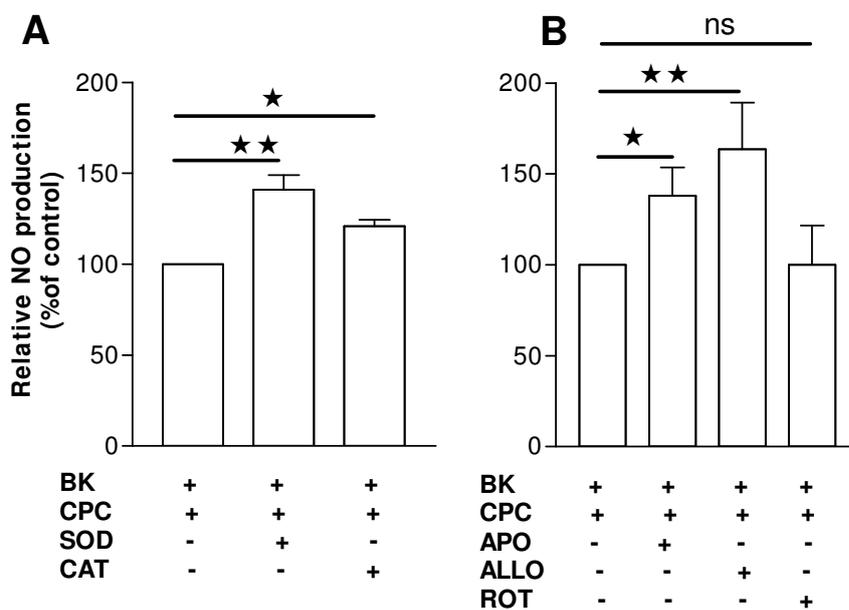
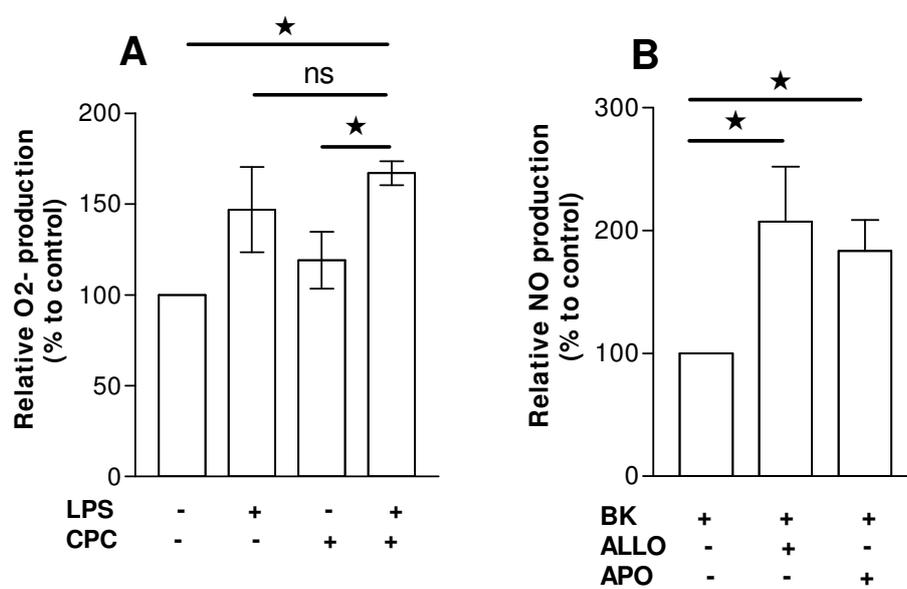


Fig. 3



**Fig. 1 : Enhanced NO production in response to BK and CPC was prevented by the nitric oxide synthase inhibitor, N<sup>w</sup>nitro-L-arginine methyl ester, L-NAME.** EAhy cells were pre-incubated with 40μM BK for 15 min before the treatment with CPC (0.03 mg/ml, 0.5% glucose) for 30 min at 37°C in the presence of 0.1 mM L-NAME, remained present throughout BK and CPC incubation. After the addition of Fe(II)Diethyldithiocarbamate(DETC)<sub>2</sub> complex, EAhy cells were then incubated for 45 min for the detection of the electron paramagnetic resonance [Fe(II)NO(DETC)<sub>2</sub>] complex by ESR. Results are expressed as the mean ± SEM of experiments performed in duplicate on 4 separate occasions. \*, p < 0.05 ; \*\*, p < 0.01 (one-way ANOVA).

**Fig. 2 : (A) The potentiate NO production following BK and CPC exposure was enhanced by SOD and Catalase.** EAhy cells were pre-incubated with 40μM BK for 15 min before the treatment with CPC (0.03 mg/ml, 0.5% glucose) for 30 min at 37°C in the presence of either 100 UI/ml SOD or Catalase, remained present throughout BK and CPC incubation. **(B) The potentiate NO production following BK and CPC exposure was modulated by inhibitors of endogenous sources of ROS.** EAhy cells were pre-incubated with 40μM BK for 15 min before the treatment with CPC (0.03 mg/ml, 0.5% glucose) for 30 min at 37°C in the presence of either 50μM allopurinol (ALLO), 2 mM apocynin (APO) or 5μM rotenone (ROT), remained present throughout BK and CPC incubation. NO level was quantified after ESR measurement. Results are expressed as the mean ± SEM of experiments performed in duplicate on 4 separate occasions. ns , not significant ; \*, p < 0.05 ; \*\*, p < 0.01 (one-way ANOVA).

**Fig. 3 : (A) Enhanced O<sub>2</sub>- production in response to LPS and CPC.** EAhy cells were pre-incubated with 40μM BK for 15 min before the treatment with CPC (0.03 mg/ml, 0.5% glucose) for 30 min at 37°C. After the addition of CMH solution, EAhy cells were then incubated for 45 min for the detection of the electron paramagnetic resonance CM-nitroxide complex by ESR. **(B) The NO production following BK exposure was enhanced after inhibition of either NADPH oxydase or Xanthine oxidase system.** EAhy cells were incubated with 40μM BK for 15 min before the treatment with 50μM allopurinol (ALLO) or 2 mM apocynin (APO) for 30 min at 37°C. NO level was quantified after ESR measurement. Results are expressed as the mean ± SEM of experiments performed in duplicate on 4 separate occasions. ns, not significant ; \*, p < 0.05 (one-way ANOVA).

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