# Detection of superoxide anion release by endothelial cells using reduced cytochrome c UV-visible absorption, ESR spin trapping, hydroethidine fluorescence and lucigenin-enhanced chemiluminescence techniques

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**Reduced cytochrome c UV-visible absorption, ESR spin trapping, hydroethidine fluorescence and lucigenin-enhanced chemiluminescence techniques, were used to study $O_2^-$ production from A23187-stimulated bovine aortic endothelial cells (BAEC). All detected $O_2^-$ release but some lack of sensitivity and specificity in probes and enzymes occurred. Cytochrome c prepared with trichloroacetic acid (TCA), TCA-free or acetylated (C2506, C7752, C4186) provided similar superoxide-dismutase (SOD)-inhibitable absorbances at 20 µM. At a higher concentration (100 µM) TCA-prepared cytochrome c could no longer detect $O_2^-$ production whereas TCA-free cytochrome c detected less $O_2^-$ at 100 µM than at 20 µM. Catalase C-40 was not contaminated with SOD-like impurities. Lucigenin provided high artefactual amounts of $O_2^-$ production from stimulated BAEC in the presence of NADH or NADPH. Moreover, even at a low concentration (5 µM) lucigenin and NADH (or NADPH) decreased endothelium-dependent relaxation induced by acetylcholine to 50 %. The use of lucigenin is not recommended.**

## Abbreviations

BAEC, bovine aortic endothelial cells;
DMPO, 5,5-dimethyl-1-pyrroline-N-oxide;
ESR, Electron spin resonance;
TCA, trichloroacetic acid;
NADPH, nicotinamide adenine dinucleotide phosphate;
SOD, superoxide dismutase.

## Introduction

Oxygen-derived free radicals play a major role in the physiology and pathology of the vessel wall [1]. To assess superoxide anion production ($O_2^-$) from endothelium, four techniques are mainly used: reduced cytochrome c UV-visible absorption [2,3], ESR spin trapping [4,5], hydroethydine fluorescence [6] and lucigenin-enhanced chemiluminescence [7,8]. The simplest technique which has been used for the longest time consists in following the reduction of cytochrome c by $O_2^-$, but the nature and the type of the cytochrome c used is not always mentioned. A few studies employing ESR and hydroethidine fluorescence have been reported. The most sensitive method commonly used appears to be lucigenin-enhanced chemiluminescence, but its reliability was recently questioned because reduced lucigenin can itself generate $O_2^-$ [9,10]. In the literature, generally only one of these techniques is used for any publication and sometimes results diverge. In the present review we compare these four methods [5,11,12] to measure the $O_2^-$ released from A23187-stimulated BAEC using the electron donors NADH and NADPH.

## Results and discussion

### *Reduced cytochrome c UV-visible absorption*

As the most currently used concentration of cytochrome c was 100 µM, we first used 100 µM commercial cytochrome c prepared with TCA [12]. Only traces of reduction were observed (Fig. 1). Curiously, after diluting cytochrome c, the SOD-inhibitable absorbance increased to 0.040. This result looked strange because generally, when a large concentration of probe is used, the highest intensity of the signal is obtained, excepted if a plateau is reached. But in this case, the signal decreased 10-fold when the concentration of the probe increased 5-fold. Using acetylated cytochrome c both




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20 µM and 100 µM concentrations gave similar absorbance (0.044) [12]. TCA-free cytochrome c gave intermediate results, an absorbance of 0.042 and 0.025 for 20 µM and 100 µM respectively. Whatever the different types of cytochrome c used, the best result was always provided with the smallest concentration: 20 µM [12]. The electron donors NADH (100 µM) and NADPH (100 µM) increased the cytochrome c signal 2.0 and 1.6-fold respectively (Fig. 2).

### *ESR signal using DMPO as spin trap*

ESR techniques, using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as spin trap, revealed $O_2^-$ production from A23187-stimulated BAEC. As the use of both SOD and catalase was required to validate the $O_2^-$ ESR signal [13,14], the lack of SOD impurities in three commercial catalase batches was tested (C-40, C-9322, C-30) [11]. Using the xanthine/xanthine-oxidase superoxide generating system, the ESR control signal decreased by 50 % in the presence of catalases C-9322 and C-30 (Fig. 3). Only catalase C-40 was not contaminated with thermolabile SOD-like antioxidants [11]. Catalase C-40 was used to check the ESR study of $O_2^-$ release by post-confluent stimulated BAEC [5,11]. NADH (100 µM) and NADPH (100 µM) increased the ESR signal 2.1 and 1.5-fold respectively. These signals were completely inhibited by SOD.

### *Hydroethidine fluorescence*

The SOD-inhibitable hydroethidine signal from 3 µM A23187-stimulated BAEC provided similar intensities to those observed with ESR using DMPO as spin trap (Fig. 2) [12]. NADH (100 µM) and NADPH (100 µM) increased the hydroethidine fluorescence 1.8 and 1.5-fold respectively. SOD-inhibitable reduced cytochrome c absorbance, superoxide ESR signal and hydroethidine fluorescence increased with the same order of magnitude (2-fold) when treated with NADH or NADPH (Fig. 2) [12].

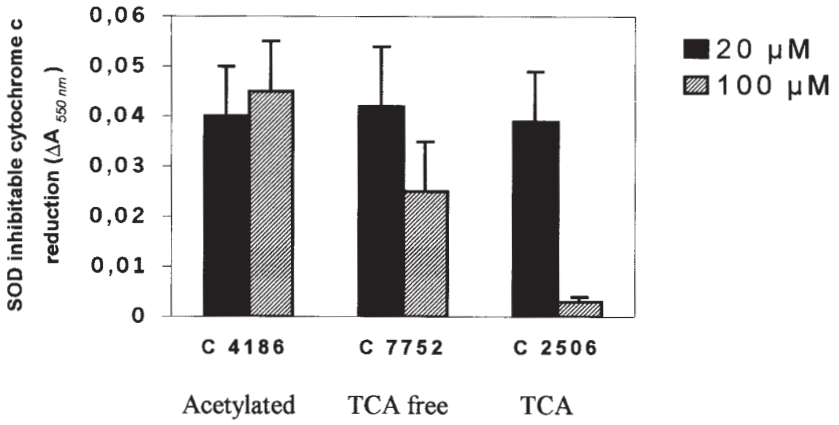


**Figure 1. SOD inhibitable cytochrome c reduction by A23187 (10 µM) stimulated BAEC (Δ absorbance / $10^6$ cells for 15 min). Cells were in confluence for 4 days. Three kinds of Sigma$^R$ cytochrome c were used : prepared with TCA (C 2506), without TCA (C7752) or with acetylation (C4186). Reproduced with the permission of the authors [12].**

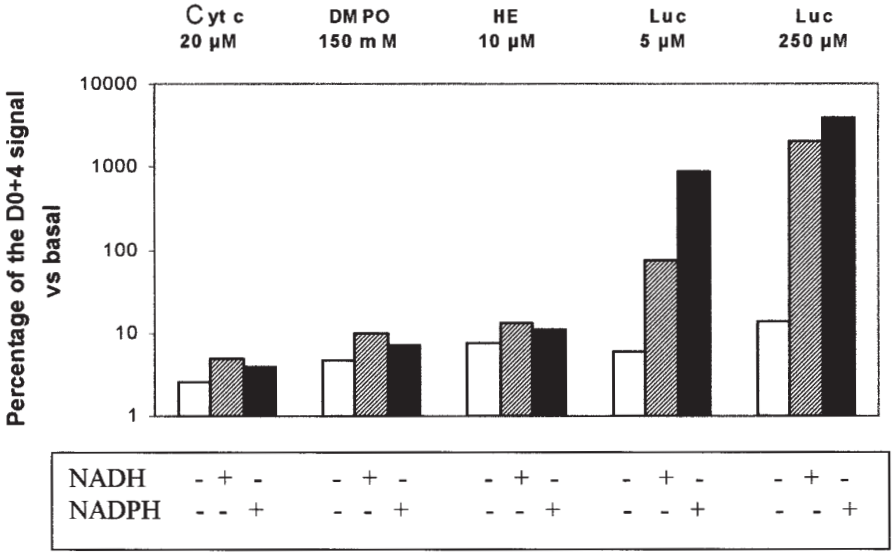


**Figure 2. Effect of NADH (100 µM) and NADPH (100 µM) on the intensity of reduced cytochrome c UV-visible absorption (20 µM), ESR DMPO-superoxide signal, hydroethydine fluorescence and lucigenin-enhanced chemiluminescence from A23187 (3 µM)-stimulated BAEC (2.5 $10^6$ cells). Reproduced with the permission of the authors [12].**

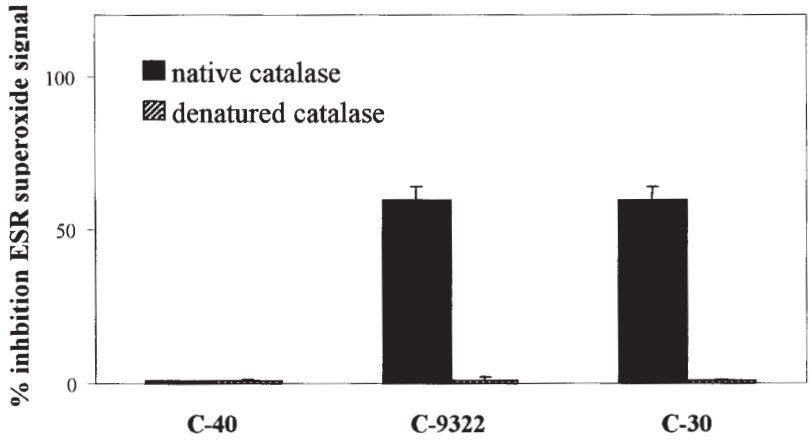


**Figure 3. Effect of different commercial solutions of catalase on the ESR superoxide signal intensity generated by the xanthine/xanthine-oxidase system. Reproduced with the permission of the authors [11].**

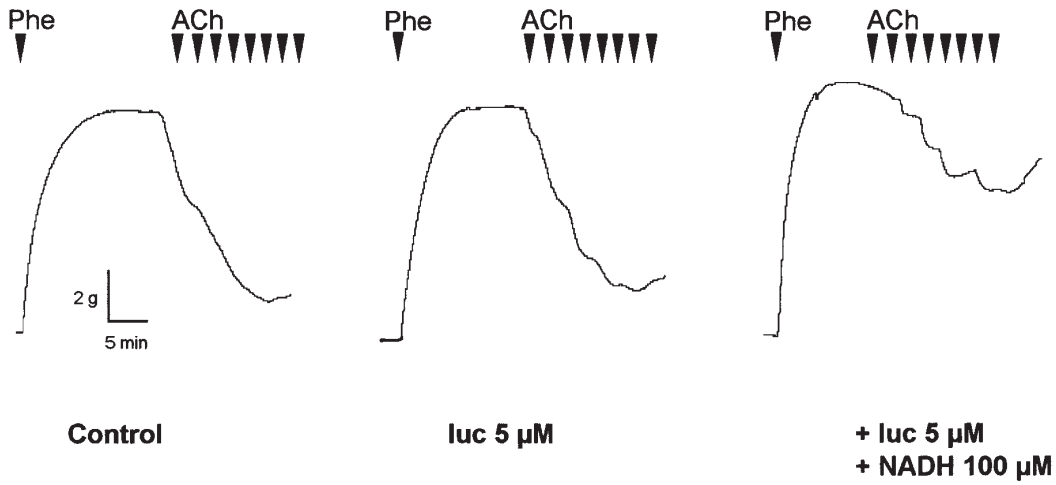


**Figure 4. Effect of lucigenin (5 µM) or lucigenin (5 µM) coincubated with NADH (100 µM) on the optimal tension of aortic ring during relaxation {acetylcholine (Ach)} after precontriction {phenyephrine(Phe)}. Arrows indicates increasing doses.**

### *Lucigenin-enhanced chemiluminescence*

#### *Production from A23187 (3 µM) stimulated BAEC*

Recently, the use of lucigenin became a matter of controversy. Lucigenin reduction by enzymes such as glucose oxidase, aldehyde oxidase or endothelial nitric oxide synthase were described to produce $O_2^-$, indicating that lucigenin is not a reliable indicator of $O_2^-$ [9,10]. However, low concentrations of lucigenin (5 µM) instead of high amounts (250 µM) appeared sensitive and sufficiently valid to allow $O_2^-$ measurement [15,16]. In our study we used both 250 µM and 5 µM lucigenin to detect $O_2^-$ production from 3 µM A23187-stimulated BAEC and to study the effects of NADH (100 µM) and NADPH (100 µM) [12]. Lucigenin (5 µM) allowed $O_2^-$ detection with similar intensity (percentage of the signal *versus* basal) to that provided by cytochrome c reduction or ESR technique (Fig. 2). Lucigenin 250 µM led to $O_2^-$ production 2-fold greater than that observed with 5 µM. The co-factors NADH (100 µM) and NADPH (100 µM) dramatically increased both 5 µM and 250 µM lucigenin-enhanced chemiluminescence in post-confluent cells: about 74 and 863-fold and 500 and 1000-fold respectively. These data suggest that, irrespective of the lucigenin concentrations, 5 µM or 250 µM, lucigenin is the main source of $O_2^-$ production under these conditions [12].

#### *Endothelium dependent relaxation of rabbit thoracic aortic ring*

In order to investigate *ex-vivo* the effect of both 5 µM lucigenin and 100 µM NADH (or 100 µM NADPH), measurements of the isometric tension of vascular rings precontracted with phenylephrine were assessed (Fig. 4) [12]. Whereas lucigenin alone (5 µM) or NADH alone (100 µM) did not affect the relaxation induced by acetylcholine, the

coincubation of 5 µM lucigenin and 100 µM NADH significantly decreased the maximal relaxation to 60 % of the control [12]. Similar results were obtained with 100 µM NADPH.

## Conclusion

Reduced cytochrome c UV-visible absorption using no more than 20 µM cytochrome c, ESR spectroscopy (using DMPO as spin trap) and hydroethidine fluorescence appear to be reliable techniques to assess extracellular $O_2^-$ release from stimulated endothelial cells. By contrast, lucigenin-enhanced chemiluminescence revealed artefactual $O_2^-$ detection in the presence of NADH or NADPH even at the low level of 5 µM.

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