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Pitfalls of Using Lucigenin in Endothelial Cells: Implications for NAD(P)H Dependent Superoxide Formation

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Since an increased endothelial superoxide formation plays an important role in the pathogenesis of endothelial dysfunction its specific detection is of particular interest. The widely used superoxide probe lucigenin, however, has been reported to induce superoxide under certain conditions, especially in the presence of NADH. This raises questions as to the conclusion of a NAD(P)H oxidase as the major source of endothelial superoxide. Using independent methods, we showed that lucigenin in the presence of NADH leads to the production of substantial amount of superoxide (~ 15-fold of control) in endothelial cell homogenates. On the other hand, these independent methods revealed that endothelial cells without lucigenin still produce superoxide in a NAD(P)H-dependent manner. This was blocked by inhibitors of the neutrophil NADPH oxidase diphenyleniodonium and phenylarsine oxide. Our results demonstrate that a NAD(P)H-dependent oxidase is an important source for endothelial superoxide but the latter, however, cannot be measured reliably by lucigenin.

Keywords: Superoxides, lucigenin, NAD(P)H oxidases, endothelium, physiology, chemiluminescence

INTRODUCTION

There is evidence that superoxide anions play an important role in the pathogenesis of vascular dysfunction and cardiovascular diseases. This is mainly due to an inactivation of NO and abolition of its vascular protecting effects.[1,2] Moreover, the reaction of superoxide with NO leads to the formation of peroxynitrite which might promote arteriosclerosis and induces apoptosis.[3,4] Several investigators pointed out that in vascular diseases, the main sources of superoxide are not circulating phagocyte cells but rather cells of the vascular wall.[2,5] With respect to its potential to interfere directly with NO release, the superoxide formation by the endothelial cells is of particular interest.[2,5–7]

For specific detection of cellular superoxide formation in cardiovascular research, the

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chemilumigenic dye lucigenin (Luc) is still being frequently used because of its sensitivity and advantage of easy handling.[8,9] Recently, however, it has been demonstrated in cell-free systems that when isolated oxidoreductases were co-incubated with Luc and the electron donor NADH, Luc itself could produce superoxide via autoxidation of its radical.[10,11] This raises important methodological questions as to earlier studies postulating that, based on NAD(P)H-dependent superoxide formation, a NAD(P)H oxidase should be the major source of superoxide in endothelial cells.[8,9,12] We therefore examined whether and in which concentration Luc could be used as a valid probe to determine the superoxide production in endothelial cell lysates containing unknown amounts of various cellular oxidoreductases, and whether there is functional evidence for a NAD(P)H-dependent superoxide formation in endothelial cells.

MATERIALS AND METHODS

Materials

2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-a]pyrazin-3-one (MCLA) was obtained from TCI Tokyo Kasei Kogyo Co., Ltd, Japan; 8-Amino-5-chloro-7-phenylpyridol(3,4-d)pyridazine-1,4(2H,3H)dione (L-012) was a generous gift from Dr. K. Schoenafinger (HMR, Hoechst AG, Frankfurt). Superoxide dismutase (SOD) was obtained from Boehringer MA, endothelial cell growth medium from PromoCell. All other compounds were obtained from Sigma.

Cell Culture and Preparations

Human umbilical vein endothelial cells were isolated from fresh umbilical cords by dispase digestion and grown to confluence in Medium 199, supplemented with 16% newborn calf serum and 20% endothelial growth medium. Confluent cells were harvested and resuspended in lysis buffer (20 mM phosphate buffered saline [PBS], 1 mM EDTA, 5 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, pH 7.0) and kept overnight on ice. The protein content was determined by the method of Bradford *et al.*

Measurement of Superoxide Generation in a Cell-Free System

The amount of superoxide, generated by the xanthine (X)/xanthine oxidase (XO) system, was assessed in parallel by the chemilumigenic probes Luc, MCLA,[13] L-012[14] and spectrophotometrically using the SOD inhibitable cytochrome c (CytC) method.

In chemiluminescence- (CL) assays the standard reaction mixture contained 100 μM xanthine (or NADH), 250 μM Luc or 10 μM MCLA or 100 μM L-012 in 400 μl PBS (pH 7.0). The superoxide specific CL was initiated by the addition of various concentrations of XO (1–5 mU) to the reaction mixture at room temperature. The intensity of the signals were recorded with a luminometer (Lumat LB 9507, Berthold) and expressed as Relative Light Units (RLU).

The CytC reduction was initiated by addition of XO to the reaction buffer containing 100 μM xanthine (or NADH), CytC (40 μM) in the presence or absence of 200 U/ml SOD. The reduction of CytC was measured by reading the absorbance at 550 nm (Ultrospec 2000, Pharmacia). The superoxide dependent reduction was calculated using the extinction coefficient of CytC ($\varepsilon_{550\,nm} = 21\,mM^{-1}\,cm^{-1}$).

Measurement of NAD(P)H-Dependent Superoxide Generation in Endothelial Cell Lysates

Chemiluminescence assays Aliquots of lysates (5 μg protein content) were co-incubated with the respective chemilumigenic dyes Luc (250 μM) or MCLA (10 μM) and the NADH (100 μM) induced CL signal was measured for 300 s.

CytC reduction The CytC (60 μM) reduction was measured in aliquots of cell lysates

(5 μg protein content) after addition of NADH (100 μM) as described above. In the presence of reducing agents, such as NADH, a substantial part of CytC is reduced directly without participation of superoxide (80–90% of total CytC reduction under our experimental conditions). However, in experiments with partially acetylated CytC, which is more sensitive to superoxide, revealed similar amount of superoxide production ($n = 3$, data not shown).

SOD sensitive nitroblue tetrazolium (NBT)-reduction (Dot Blot) As another independent index of superoxide generation, the SOD sensitive part of the nitroblue tetrazolium (NBT) reduction was determined.[15] Cell lysates (5 μg protein content) were transferred onto nitrocellulose membranes (Schleicher & Schuell), placed into a reaction mixture (2.7 mg NBT in 3 ml PBS ± SOD 200 U/ml) and the reaction was started by adding of NADH (100 μM). The intensity of the visible blue formazan precipitate was analyzed by densitometry using a commercially available software (Molecular analyst) and corrected for the SOD insensitive background and expressed in arbitrary units.

Statistical Analysis

Statistical comparisons between controls and treatments in the same experimental group were performed with the Wilcoxon signed rank test for paired observations. Comparisons between groups were performed using the Kruskal–Wallis-test. Differences were considered significant at an error probability of $p < 0.05$. All results are expressed as means ± SEM.

RESULTS

Detection of Superoxide Production in X/XO System

The different superoxide assay systems were first evaluated using the X/XO system as source of superoxide. In the presence of 100 μM xanthine, the superoxide-dependent signals correlated well with the concentration of XO (1–5 mU) used ($r^2 > 0.98$ for CytC-, MCLA-, L-012- and Luc-assay). In all CL-assays, SOD (200 U/ml) completely abolished the X/XO-induced superoxide induced signals.

Superoxide Production by Lucigenin in the Presence of NADH

To estimate the superoxide formation due to autoxidation of the Luc radical in the presence of NADH we studied the effect of Luc in an isolated superoxide generating system. Co-incubation of NADH (100 μM) and XO (5 mU) led to a low superoxide formation (~0.23 μM/5 min), as measured in CytC-assay, and increased the L-012-CL by 4.8-fold over its background signal (Figure 1A and B). The additional administration of xanthine (100 μM) to NADH/XO system strongly augmented the initial superoxide signal in CytC- and L-012-assay by further 46.4- and 52.6-fold, respectively (Figure 1A and B). In contrast, the NADH/XO system significantly increased the Luc-CL 19.1-fold over its background signal, whereas additional application of xanthine further amplified the Luc-CL only 3.8-fold (Figure 1C). These results indicate that the Luc-CL was already markedly increased by the NADH/XO system which induces a Luc-radical mediated superoxide formation.

Lucigenin Mediates Superoxide Formation in Endothelial Cell Lysates

To assess whether there is a non-specific superoxide formation by Luc in endothelial cell lysates as well, we measured the NADH-dependent superoxide generation in the presence and absence of additional Luc using different methods. As shown in Figure 2A, the MCLA-CL was significantly increased by 20% after addition of NADH (100 μM) to the cell lysates. The addition of Luc (250 μM) to this system markedly enhanced the MCLA-CL 2-fold over its background control

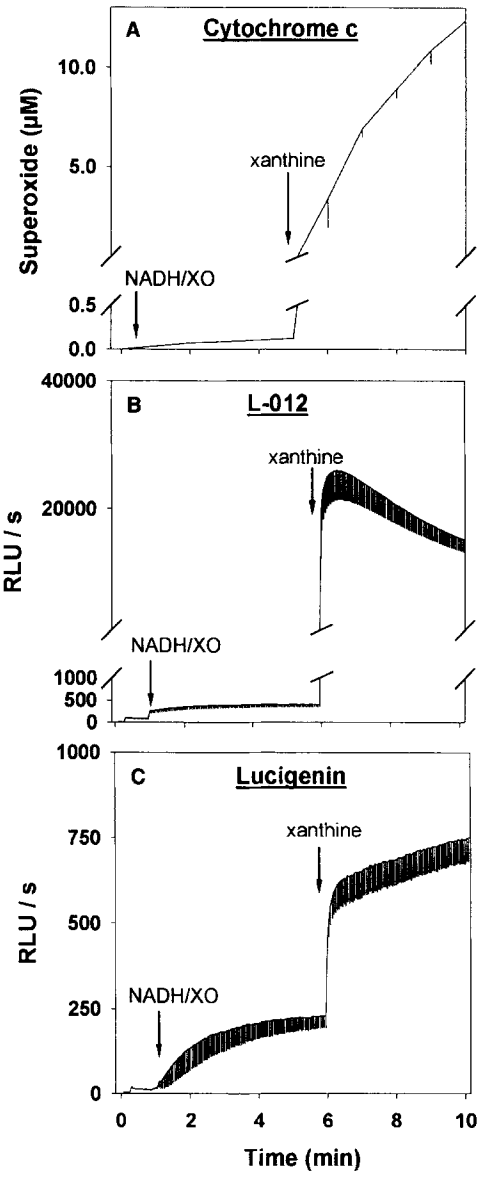

FIGURE 1 A–C: Luc-mediated superoxide formation in NADH/XO system. The superoxide formation in NADH/XO (100 μM/5 mU) was studied using three independent superoxide assays: (A) CytC- (40 μM), (B) L-012- (100 μM), and (C) Luc-assay (250 μM) ($n = 4$ each). After 5 min xanthine (100 μM) was added to the reaction mixture to compare the superoxide-dependent signals induced by NADH/XO and X/XO system.

signal ($n = 11$, $p < 0.01$) which was completely sensitive to SOD (Figure 2A). This effect of Luc cannot be attributed to a Luc-derived CL since the signal intensity of Luc was ~100-fold lower compared to MCLA, as determined in X/XO system (Table I). It has been recently postulated that 5 μM Luc would not result in a significant redox cycling.[16] Therefore, we tested the effect of this low concentration of Luc on superoxide formation in cell lysates. Luc at a concentration of 5 μM still elicited a significant increase in MCLA-CL by 12% (0.82 ± 0.05 vs. $0.92 \pm 0.05 \times 10^6$ RLU/10 μg protein/300 s, $n = 8$, $p < 0.05$), although when used alone, the signal to noise ratio was too low for sensitive measurements of superoxide (unpublished observation). A Luc-mediated superoxide formation in cell homogenates was also observed in the NBT-assay (Figure 2B). Incubation of lysates with NADH resulted in blue staining which was partially inhibitable by SOD (~30% under our experimental conditions). In the presence of Luc (250 μM), this SOD-sensitive NBT reduction was markedly enhanced by 2.8-fold ($n = 11$, $p < 0.05$), whereas the SOD independent part was not affected.

Evidence for Luc Independent Superoxide Production in Endothelial Cell Lysates

In view of the marked autoxidative effects of Luc in the presence of NADH we studied whether there was at all a significant NADH-dependent superoxide formation in endothelial cell lysates in the absence of Luc. As shown in Figure 3, addition of NADH and NADPH (100 μM each) induced a significant superoxide generation as measured with the SOD inhibitable CytC assay. Moreover, the flavoprotein enzyme inhibitor diphenyleniodonium (DPI, 100 μM, $n = 9$, $p < 0.05$) as well as pre-incubating the cells with phenylarsine oxide (1 μM, $n = 8$, $p < 0.05$), a direct inhibitor of the neutrophil NADPH oxidase, significantly reduced the NADH induced superoxide formation by 63% (86.5 ± 11 vs. 32.5 ± 8 μM superoxide/mg protein/15 min) and 66% (102.3 ± 37 vs. 35.3 ± 12 μM superoxide/mg protein/5 min), respectively.

This superoxide production in CytC-assay was compared with the superoxide signal in Luc assay

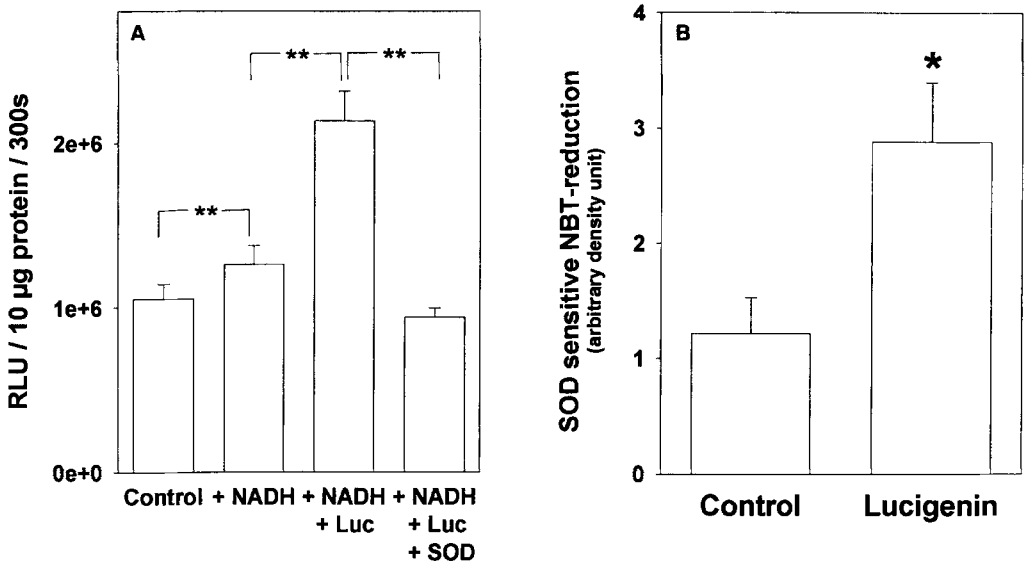

FIGURE 2 A,B: Luc-mediated superoxide formation in endothelial cell lysates in the presence of NADH. (2A) MCLA-assay: Cell lysates of endothelial cells were stimulated with 100 µM NADH and the MCLA-CL (10 µM) was determined in the absence or presence of additional Luc (Luc 250 µM, SOD 200 U/ml, $^{**}p < 0.01$, $n = 11$). (2B) NBT-assay: Dot blots of cell lysates were incubated with NADH (100 µM) and the SOD (200 U/ml) sensitive reduction of NBT was measured in the absence or presence of additional Luc (250 µM, $^{*}p < 0.05$, $n = 11$).

TABLE I Signal intensity of Luc and MCLA-CL in cell-free X/XO system

	Lucigenin (250 µM) (mean-RLU/10 min)	MCLA (10 µM) (mean-RLU/10 min)
Background signal	18.4 ± 0.2	5860 ± 62
XO (1 mU/ml)	97.6 ± 13.1	11 128 ± 47
XO (2 mU/ml)	239.1 ± 29.0	28 963 ± 88
XO (5 mU/ml)	601.6 ± 47.2	64 273 ± 84
XO (5 mU/ml) + SOD (200 U/ml)	30.4 ± 2.0	5093 ± 185

using cell lysates from identical pools and the X/XO calibration curve (Figure 4A). The Luc signal, however, was ~15-fold higher than measured in CytC-assay (Figure 4B, expected and measured Luc signal) further confirming the results in MCLA- and NBT-assay.

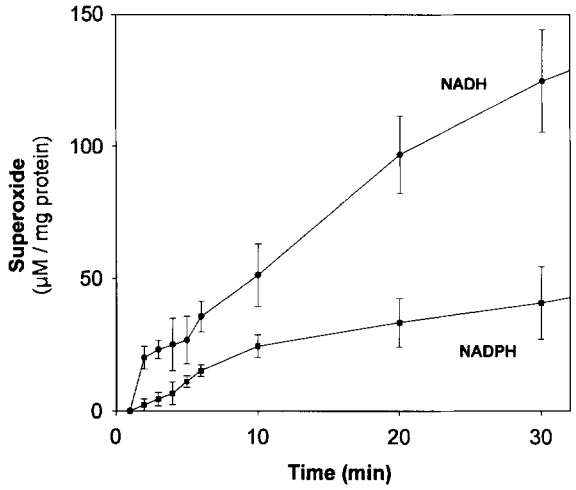

FIGURE 3 NADH/NADPH-dependent superoxide formation in endothelial cell lysates. Endothelial cell lysates were co-incubated with either NADH or NADPH (100 µM, each), and the superoxide formation measured using the SOD inhibitable CytC- (60 µM) assay. Values represent the mean ± SEM ($n = 3$).

DISCUSSION

Our results demonstrate that the Luc assay cannot be used for a valid quantitative detection of NAD(P)H-dependent superoxide production in endothelial cell lysates. In the presence of the electron donor NADH, the addition of Luc initiates a marked increase of superoxide formation in endothelial cell lysates.

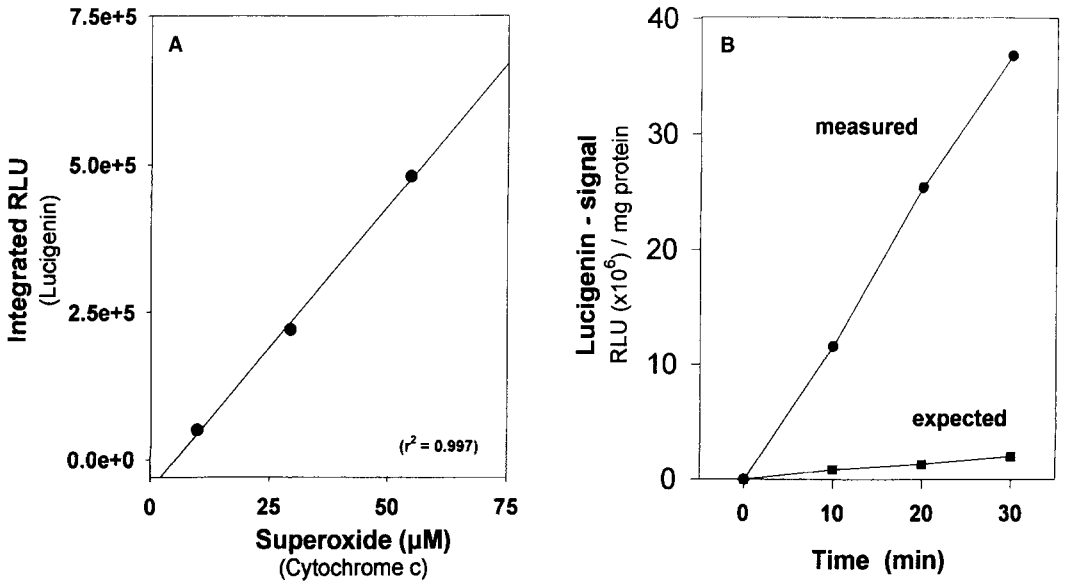

FIGURE 4 A,B: Difference between expected and measured superoxide signal in Luc-CL-assay. (4A) X/XO-calibration curve: In cell-free X/XO system there was an excellent correlation between the Luc CL and the CytC reduction (XO 1–5 mU, xanthine: 100 μM, $n = 4$). (4B) NADH-dependent superoxide formation in CytC- and Luc-assay: In cell lysates/NADH system, the calculated superoxide formation was assessed with values measured by the CytC-assay after different time points and referred to the Luc values using the X/XO calibration curve ($n = 3$). The results obtained from the CytC-assay were used to construct the "expected" Luc values.

It has been recently shown[10,11] that Luc mediates superoxide formation in enzyme solutions that normally produce little (NADH/XO) or no superoxide (glucose/glucose oxidase). To estimate the rate of Luc-mediated superoxide formation, we compared the superoxide generation in NADH/XO with X/XO system, and could demonstrate that the larger portion of the Luc-CL in NADH/XO system was due to an autoxidation of the Luc radical producing substantial amounts of superoxide. This occurred not only in isolated enzyme systems but also in endothelial cell lysates. Using alternative superoxide assays we demonstrated that Luc, in the commonly used concentration of 250 μM, considerably augmented the superoxide production. In contrast to a previous report by Li *et al.*[16] who could not detect a redox cycling of Luc at a concentration of 5 μM using spin trap techniques, the MCLA assay revealed a significant Luc-dependent superoxide formation under our experimental conditions. These findings suggest that Luc, even at this low concentration, could undergo a redox cycling in endothelial cell homogenates.

We did not examine whether Luc also mediates superoxide formation in intact endothelial cells. Afanas'ev *et al.* reported that the redox cycling of Luc is of no importance in aqueous solutions with regard to thermodynamic considerations.[17] However, it has been shown very recently that this cannot be simply transferred to a complex system such as intact cells or cell homogenates: In isolated aortic rings of rats Tarpey *et al.*[18] showed that Luc *per se* causes an impairment of endothelial function which could be completely restored by adding of SOD. These data strongly indicate that Luc, when added to a cellular system, significantly undergoes a redox cycling resulting in an artificial formation of superoxide. This is in full agreement with our results that Luc induces superoxide production in the presence of an unknown amount of cellular oxidoreductases.

The autoxidation of Luc can also not be excluded by the use of the non-specific inhibitor of several flavoprotein enzymes DPI, since it may block not only the NAD(P)H oxidase but also enzymes that mediate NADH-dependent formation of Luc radicals.[19,20]

In recent years, it became clear that superoxide radicals play an important role in the control of vascular tone either by acting directly as a vasoactive substance, or by interfering with the release or the stability of NO.[1,2] For the understanding of the pathogenic mechanisms, as well as for the development of new therapeutic approaches, it is of high interest to identify the major source of superoxide in endothelial cells. Based on the detection of subunits of the neutrophil-type NAD(P)H oxidase by immunohistochemistry and PCR, it has been postulated that this enzyme is the major source of vascular superoxide formation.[21,22] In the past, studies investigating the functional role of this enzyme in vascular cell homogenates have been almost exclusively done using Luc to monitor NADH induced superoxide formation.[8,9] Using cell lysates and the established CytC-assay, the present study now clearly demonstrates a functional role of a NAD(P)H-dependent superoxide formation in endothelial cells. Furthermore, the direct inhibitor of the neutrophil NAD(P)H oxidase, phenylarsine oxide,[23] significantly attenuated this superoxide formation. The recent demonstration that the inhibition of the NADPH oxidase assembly by specific peptides prevented free radical generation in the lung[7] and that antisense oligonucleotides against the subunit p22phox had similar inhibitory effects in vascular cells,[24,25] further supports the view that this enzyme plays a significant role in vascular superoxide formation. The latter, however, cannot be measured reliably by the Luc-assay as shown here with several independent methods.

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