

Application of Lucigenin-enhanced Chemiluminescence (LEC) in the
Evaluation of Oxidative Stress of Isolated Aortic Rings From Rats

Shih-Hsuan Chou^a, Chiu-Feng Huang^a, Ying-Ping Chang^a, Yu-Ren Wang^a,
Jin-Ye Chou^a, Chia-Hung Yen^b and Ying-Tung Lau^a

Department of Physiology and Pharmacology, Chang Gung University College
of Medicine, 259 Wen Hwa 1Rd., ^bDepartment of Plastic and Reconstructive
Surgery, Chang Gung Memorial, Hospital, Chang Gung University, 5, Fu-Hsing
St., Kwei-Shan, Tao-Yuan, Taiwan, R.O.C.

The principal investigator of this research group, Ying-Tung Lau, is professor and associate dean of the School of Medicine at Chang Gung University in Taiwan, R.O.C. He is currently the president of Chinese Physiology Society (Taipei). The major objectives of the research program include the study of gender difference of vascular responses associated with oxidative stress (SHC, YPC, CHY), the protective role of estrogen and estrogen receptor against oxidative stress (CFH), and the effects of diet control or caloric restriction on oxidative status and vascular function (SHC, YRW).

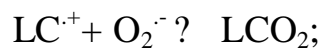
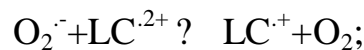
Introduction

Accumulating evidence suggests that the NADH/ NADPH oxidase may be responsible for excessive superoxide generation in several cardiovascular diseases (1) and that the activation of this source of superoxide can lead to endothelial dysfunction by reducing NO[•] bioavailability. This mechanism likely plays an important role in the genesis of vascular disease in several pathophysiological conditions (1-3). Luminometric assays, among other techniques, provides a sensitive and fast method to detect superoxide anion (O₂^{•-}) (4). The aim of this study was to evaluate the application of Plate CHAMELEONTM (Hidex, Finland) for the determination of the production of O₂^{•-} in vascular tissues by using lucigenin-enhanced chemiluminescence (LEC). We measured background (or spontaneous) photon level without tissues under various conditions including: Krebs-HEPES solution, NADH, NADPH as well as different concentrations of lucigenin. To determine biological responses, we measured LEC with or without NADH/NADPH in the presence of aortic tissues derived from healthy rats. Streptozotocin (STZ)-induced hyperglycemia, a known pathological condition that involves oxidative stress, was examined and the nature of NADPH-dependent LEC investigated.

Materials and methods

2.1 Lucigenin-enhanced chemiluminescence (LEC)

Levels of superoxide production from aortas were determined using the LEC with a luminometer (Plate CHAMELEONTM, Hidex, Finland). The monitor system consists of a lightproof box, a photon multiplier, and a photon counter. Lucigenin, an acridylum dinitrate compound that emits light on reduction and interaction with superoxide anion, was used to measure the production of vascular superoxide (2,4,5). The relevant reactions of the assay are as follows (LC= lucigenin):



The flux of photons (h ν) emitted is measured in terms of LEC intensity and taken as superoxide anion levels.

2.2 Vascular preparation and testing conditions

After the removal of aortic arteries from adult male rats, the vessels were immediately rinsed in Krebs-HEPES solution of the following composition (in mmol/L): 99.01NaCl, 4.69KCl, 1.87CaCl₂, 1.20MgSO₄, 1.03K₂PO₄, 20Na-HEPES and 11.1glucose, and bubbled with 95% O₂ and 5% CO₂, pH 7.4. Adherent connective and fat tissue of the vessels were cleaned and cut into rings approximately 2 mm in length and then incubated for a 20 minutes equilibration period (95% O₂ and 5% CO₂). During the measurement of background (or spontaneous) photon level without tissues, the effects of NADH, NADPH and lucigenin (two concentrations) on LEC were examined.

Firstly, the background photon level was measured in a white 96-well plate (OptiPlateTM-96, PerkinElmer). Then, lucigenin (5 or 200 μ M) and 100 μ M

(final concentration) of NADH or NADPH in 200 μ L of Krebs-HEPES solution was separately added to each well and the output of photon level was measured. Next, aortic rings were transferred to the 96-well plate (a single well contained only one ring) consisted of Krebs-HEPES solution with or without lucigenin (5 or 200 μ M) and the output of LEC was measured again. Finally, NADH or NADPH was added by auto-injection (dispenser mode) to each well and the output of the LEC (with or without lucigenin) was measured. Measurements were taken in turn for each well and expressed as counts per second (CPS).

2.3 STZ-induced hyperglycemic rats

A single tail vein injection of STZ (55 mg/kg; Sigma Chemical Co., St. Louis, MO) was given to adult rats one day before they were sacrificed. These rats developed increased serum glucose concentration at the time of experiment (Chang and Lau, unpublished). Control animals received an equivalent volume of citrate buffer (50mM, vehicle). Aortic arteries were then removed from the STZ-rats and LEC determined with or without NADPH as described before.

In addition, the possible enzymatic source of superoxide in the vessels was determined pharmacologically (30-min preincubation) in the presence or absence of diphenylene iodonium (DPI, 10 μ M), a selective inhibitor of flavin-containing enzymes including NA(D)PH oxidase.

Results and Discussion

We routinely observed very low level of LEC (< 60 cps) with empty plate and that this LEC level was not altered by adding Krebs-HEPES alone or together with lucigenin (5 or 200 μM), NADPH (100 μM), or NADPH (100 μM). Combination of lucigenin with NADH or NADPH did not change the LEC reading as well in the absence of aortic rings (data not shown). It also remained unchanged with the addition of an aortic ring with Krebs-HEPES solution containing NADH/NADPH in the well (Fig. 1). Together, these observations indicate a low and stable LEC reading without lucigenin in the presence of various compounds usually employed to study vascular response. However, lucigenin (5 μM) caused a small but significant increase of LEC ('basal' level), high concentration (200 μM) of lucigenin caused a large increase which could be partially due to redox cycling (2,5). Following the addition of NADH or NADPH to each well, LEC increased significantly when compared with 'basal', this is termed NADH/NADPH-stimulated LEC reflecting an induced O_2^- production associated with NADH/NADPH oxidase (3-5). Inhibitors of potential O_2^- sources including oxypurinol (100 μM) for xanthine oxidase, indomethacin (10 μM) for cyclooxygenase, N^0 -Nitro-L-Arginine (100 μM) for (uncoupled) NO synthase, rotenone (10 μM) for mitochondria electron transfer chain, and DPI (10 μM) were tested and significant inhibition was only found with DPI (data not shown). O_2^- scavengers including both superoxide dismutase (300 U/mL) and Tiron (10 mM) could significantly reduced the NADPH-stimulated LEC intensity (data not shown). The NADPH-induced response was nearly 10-fold stronger than that of NADH with low lucigenin (5 μM). In the presence of NADH/NADPH high lucigenin concentration (200 μM) was found to result a much larger LEC signal, probably redox cycling in

nature.

Figure 2 depicts a typical trace of LEC recording (with 5 μ M of lucigenin) detected in rings derived from STZ-rats and control rats. We found that basal LEC (open symbols) was only slightly increased in rings from STZ-induced hyperglycemic rats compared to control. However, NADPH-stimulated LEC was significantly higher in rings from STZ-induced hyperglycemic rats compared to control. The NADPH response was very rapid (≤ 1 second) and lasted for more than 1h. Furthermore, these increases of NADPH-stimulated LEC were inhibited by DPI, a non-specific NADPH oxidase. These results were consistent with our recent findings that sepsis-induced O_2^- production is mainly due to NADPH oxidase activation and that propofol ameliorates such O_2^- production as well as sepsis-induced vascular dysfunctions (3). We thus conclude that with low lucigenin concentration (5 μ M), NADPH-stimulated LEC as determined herein provides an effective assessment for O_2^- production from NADPH oxidase in aorta of rats under pathological conditions.

Discussion

In our experimental circumstances (with or without lucigenin and NADH/NADPH), the generation of photon level did not differ from background in the absence of aortic tissues. Moreover, our data indicate that the generation of LEC significantly augmented only in the presence of aortic tissues (when incubated with lucigenin). In concordance of previous studies [5,6], NADH is the preferred substrate in the concentration of 5 μM -lucigenin and NADPH is the preferred substrate in the concentration of 200 μM -lucigenin. Furthermore, this system successfully distinguished the differences of chemiluminescence between the control and STZ-induced hyperglycemic rats. We conclude that Plate CHAMELEONTM displays a significantly biological availability and provides an accurate assessment on the detection of the superoxide production in vascular tissue.

References

1. Guzik TJ, West NE, Black E, McDonald D, Ratnatunga C, Pillai R, and Channon KM (2000) Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. *Circ Res.* 86:E85–E90.
2. Pagano PJ (2001) NAD(P)H oxidase: Marker of the dedifferentiated neointimal smooth muscle cell? *Arterioscle Thromb Vasc Biol.* 21: 175-177.
3. Yu HP, Lui PW, Hwang TL, Yen CH and Lau YT (2005) Propofol improves endothelial dysfunction and attenuates vascular superoxide production in septic rats. *Critical Care Medicine.* (in press)
4. Brandes RP and Janiszewski M (2005) Direct detection of reactive oxygen species ex vivo. *Kidney International.* 67:1662-1664.
5. Munzel T, Igor B, and Harrison DG (2002) Detection of superoxide in vascular tissue. *Arterioscle Thromb Vasc Biol.* 22: 1761-1768.

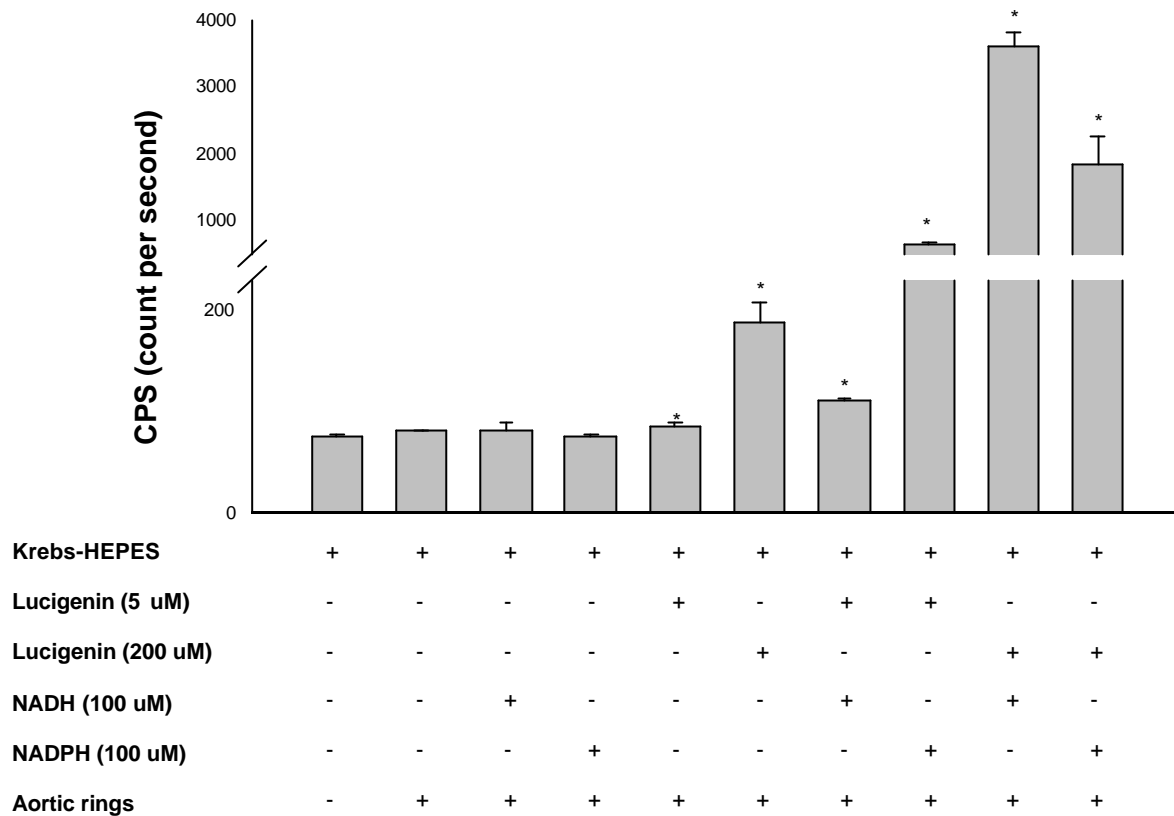


Figure 1. superoxide anion measurement (in CPS) of aortic rings in the presence or absence of lucigenin and / or NADH/NADPH. Values are means \pm SEM, * $p < 0.05$ vs. Krebs-HEPES alone.

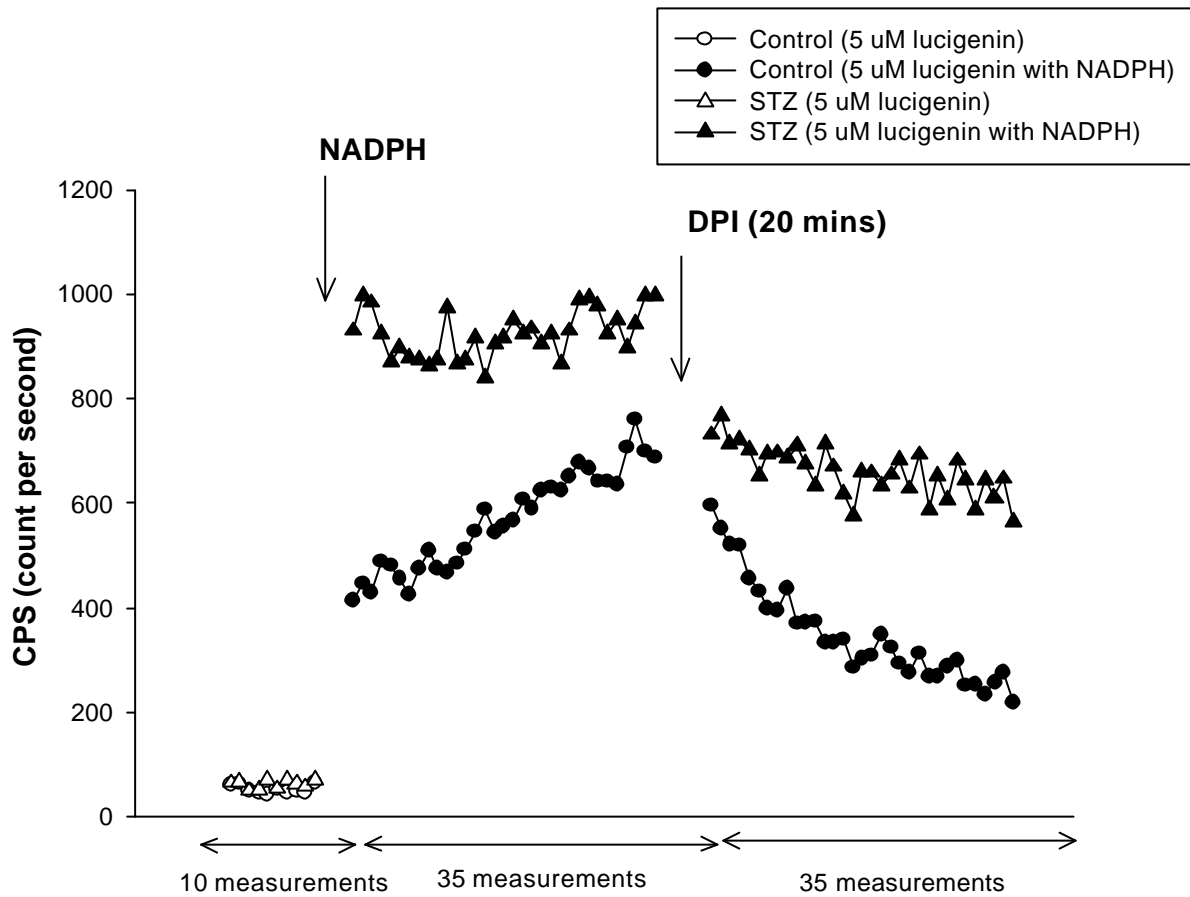


Figure 2. NADPH (100 μ M)-induced LEC (in CPS) in aortic rings derived from STZ-induced hyperglycemic rat (open and solid triangles) and control rat (open and solid circles)