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Characterization of the $\cdot O_2^-$-Formation by Pyridine Nucleotide in Rat Hepatocytes

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Abstract: The detection with lucigenin under physiological conditions is selective for $\cdot O_2^-$, for it can be accepted that lucigenin indicates actual intramembranal $\cdot O_2^-$-formation. Lucigenin chemiluminescence (CL) was elicited from the plasma membrane (PM) only by addition of reduced pyridine nucleotide. NADPH was preferred to NADH in PM and hepatocytes. This specificity was masked by $NAD(P)^+$ inhibition. The half maximum rate of CL increase was obtained with 1.5 μM NADH or 55 μM NADPH in hepatocytes and 6 μM NADH or 30 μM NADPH in plasma membranes. Measurement of these NADPH values required the presence of a NADPH-regenerating system. With NADPH the maximal rate obtained was 10 fold higher than with NADH. NADPH and NADH could produce CL when having access from either side of the membrane. They seemed to react with the identical acceptor because NADH-induced CL was also inhibited by $NADP^+$. The characteristics of $\cdot O_2^-$-formation produced by pyridine nucleotide will be discussed.

Key words: chemiluminescence, NAD(P)H, NADPH-regenerating system, $\cdot O_2^-$-formation, plasma membrane, rat hepatocytes.

$\cdot O_2^-$-formation in phagocytes and in injury occurring in the "pro-oxidant state" is always exofacially from membrane surface to the surrounding aqueous phase (Crane *et al.*, 1985). However we found $\cdot O_2^-$-formation in plasma membranes of hepatocytes, and later in other cell types. $\cdot O_2^-$-formation was not sensitive to superoxide dismutase (SOD) and was not detectable by the usual detection methods for $\cdot O_2^-$, such as reduction of added cytochrome c or cyanoferrate, but with the amphiphilic substances like lucigenin or luminol only.

The superoxide radicals are dismutated before they reach the aqueous surrounding, where they could be detected (Wolter, 1993). H_2O_2 formed in the dismutation reaction is also not detectable outside (Wolter, 1993). Both products seem to be formed in concert to their need and to participate in the regulation of intramembranal redox process. The reactions involved are as yet unknown. It is feasible that a delicate regulation on the degree of reduction at SH-groups of membrane enzymes is fundamental to the function of intramembranal redox processes. This possibility was discussed with respect to the NADH oxidase which is known to be an essential enzyme for $\cdot O_2^-$-formation in plasma membrane (Clark *et al.*, 1981; Goldenberg, 1982).

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The differences of $\cdot O_2^-$-formation by pyridine nucleotide between hepatocytes and its plasma membrane fraction will be discussed. The other purpose of this research is to settle the questions whether both reacted with the same $\cdot O_2^-$-generating system and which nucleotide was the preferred substrate mainly on a highly selective lucigenin chemiluminescence assay. In this study, we have isolated plasma membrane from liver and hepatocytes, and then characterized $\cdot O_2^-$-formation in this system after the addition of reduced pyridine nucleotide.

Materials and Methods

Materials

Collagenase A, lucigenin, NAD(P)H, $NAD(P)^+$, and SOD were purchased from Boehringer (Mannheim, Germany). D-(U-^{14}C)-glucose was from Amersham Buchler (Braunschweig, Germany). ^{14}C-carboxydextran, and HTO were obtained from Du Pont de Nemours (Dreieich, Germany). D(+)-glucose was a product of Merck (Darmstadt, Germany).

Isolation of liver cells

Liver cells were isolated according to the procedure with a modification (Berry & Friend, 1969; Baur *et al.*, 1975). A female Wistar rat (170~200 g) was narcotized by ether. After opening the abdomen and tub-

ing of the vena cava inferior and vena portae, the liver was perfused with 1.2 mM Ca^{2+} perfusion medium I (121 mM NaCl, 18 mM $NaHCO_3$, 5.9 mM KCl, 0.74 mM NaH_2PO_4, 0.6 mM $MgSO_4$, 5 mM D-glucose, pH 7.2 at 37℃, perfusion speed: 3 $ml \times min^{-1} \times g^{-1}$ liver). All perfusion media were aerated with carbon dioxide. After opening the thorax, a tube was inserted into the proximal part of the vena cava inferior, the liver was perfused with Ca^{2+}-free perfusion medium at 37℃ via the vena portae for 20 min. Perfusion of the isolated liver was carried out with a recirculating medium containing 0.47 mM Ca^{2+}, 2 mM pyruvate and 25 mg collagenase (Boehringer collagenase A 0.21 U/mg) in 50 ml Ca^{2+}-free medium. Then, it was continued through the vena cava for 10~15 min. The liver was disintegrated after 25~30 min of perfusion. After shredding the liver, the tissue was transfered to a round-bottomed flask, and enzymic treatment (collagenase medium) was continued for another 6 min with carbon dioxide under slow rotation (in water bath at 37℃). The treated tissue was filtrated (300 μm gauze) and washed (Washing medium: 137 mM NaCl, 5.2 mM $CaCl_2$, 0.9 mM $MgSO_4$, 5 mM D-glucose, 3 mM Na_2HPO_4/KH_2PO_4, pH 7.2 at 0℃) by centrifugation 3 times (70×g, 20 sec). The cells were stored in suspension at 0℃ in a standard medium containing 137 mM NaCl, 5 mM KCl, 0.4 mM $MgSO_4$, 0.12 mM $CaCl_2$, 3 mM KH_2PO_4/Na_2HPO_4, and 20 mM morpholino-3-propanesulfonic acid (MOPS) adjusted to pH 7.3 with Tris base.

The protein concentration was 30~50 mg protein /ml and cell viability was determined by tryphan blue staining, LDH and intracellular Na^+K^+ concentration.

Hepatic plasma membranes were isolated as previously described (Emmelot *et al.*, 1974).

Incubation of liver cells

Incubation of cells was carried out at 37℃. Normal incubation medium was identical with standard medium (pH 7.3), with 1 mg cellular protein/ml. Determination of cell volume and adherent fluid volume were carried out with 9.25 KBq ^{14}C-carboxydextran and 74 KBq tritium-marked water (HTO) per 1 ml incubation medium applying the silicon centrifugation method.

The silicon centrifugation was performed according to the procedure described elsewhere (Pfaff, 1965; Klingenberg, 1967).

Determination of intracellular Na^+K^+ concentration, cellular volume and adherent fluid

D-glucose (16 mM) was added to the incubation medium (containing ^3H_2O and ^{14}C-carboxydextran) either alone (control) or together with NADH. Apart from that, the same procedures as in glucose uptake studies were used.

^{14}C-D-glucose uptake into the isolated cells

A cell suspension containing 1 mg cellular protein/ml was preincubated for 8 min at 37℃. Then ^{14}C-D-glucose (8 KBq/200 μl) and 16 mM glucose were added to the incubation medium. 200 μl samples were withdrawn after 5, 15, 25, 35, 45, and 60 seconds. The effects of NADH on uptake were compared with controls.

Lucigenin chemiluminescence (CL)

Chemiluminescence assay was carried out with lucigenin (Albrecht *et al.*, 1990; Allen, 1981, 1982). In the physiological pH range, superoxide radicals are detected selectively by lucigenin-CL. The experiment was performed at 37℃ with 1 mg/ml hepatocyte or 50 μg plasma membrane/ml in an automated luminometer (LB9500, Firma Berthold, Wilbad, Germany) interfaced with a PC-compatible computer. The program for kinetics was developed in institute of pharmacology and toxicology in Tuebingen, Germany. The lucigenin-enhanced reactions were followed by a highly sensitive photomultiplier (375~620 nm). Each polyethylene minivial contained a final volume of 1.5 ml, including buffer.

Results and Discussion

The cells had been preincubated for 1 min at 37℃ and then CL was measured simultaneously with the addition of nucleotide. I observed completely different kinetics of CL. The initial velocity of the reaction was higher with NADPH than that of NADH addition. It reached the maximum value after 137 sec. by NADH but after about 20 sec by NADPH. After NADH addition the CL decreased slowly, after NADPH quickly. The total number of photons counted, integrated by the instrument during the test time was twice as high after NADH than after NADPH addition. This difference in the yields remained significant, even though the integration was incomplete. The basal CL, amounting to about 90,000 counts in 5 min without addition of nucleotide, was not considered.

What causes the rapid restraint of CL after NADPH addition? The assumption was tested whether this could be due to the $NADP^+$ produced. A NADPH-regenerating system consisting of isocitrate and isocitrate dehydrogenase (ICDH) was therefore added (as it is commonly used in the research on cytochrome P_{450}-activity) (Fig. 1). While initial rate and the maximum value of CL were increased, the CL still dropped unex-

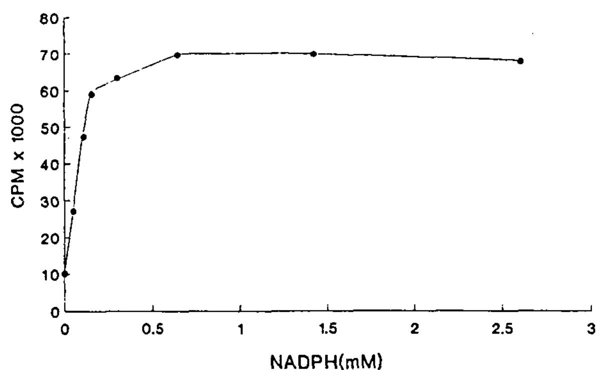

Fig. 1. Dependence of the initial rate of lucigenin-CL with hepatocytes on the NADPH concentration in the presence of NADPH-regenerating system with isocitrate and isocitrate dehydrogenase (ICDH). Incubation was carried out with 1.5 ml incubation medium, 1 mg hepatocyte protein/ml, 13.3 μM lucigenin, 10 mM isocitrate and 0.2 mg ICDH at 37℃. The CL assay was started with 1 mg hepatocyte protein/ml for over 60 sec. The initial rate was calculated from the initial linea increase of CL in each original diagram.

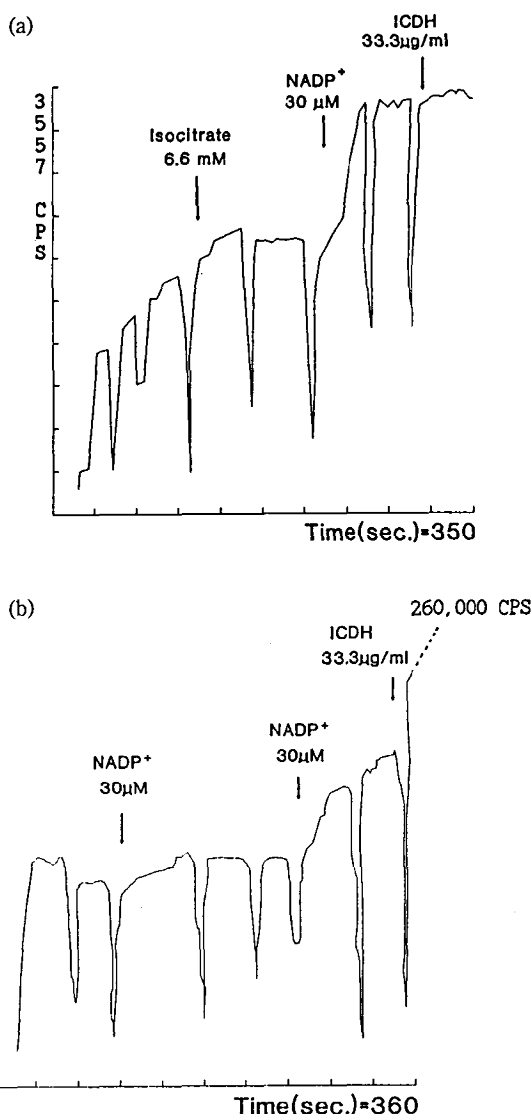

Fig. 2. Stimulation of the CL of hepatocytes by an incomplete NADPH-regenerating system. Incubation was carried out with 1 mg hepatocyte protein/ml, 13.3 μM lucigenin at 37℃. 3 mM calcium was preincubated in Fig. 2b.

pectedly thereafter. It must be assumed that, even though $NADP^+$ produced may suppress NADPH-induced CL the total amount of triphosphopyridine nucleotide might be decreased by some other process. Because the added activity of ICDH was sufficient for the continuous reduction of the produced $NADP^+$ to NADPH (Fig. 2).

Inhibition of NADPH-induced CL by $NADP^+$ shall be characterized. The decrease of initial rate of this CL in isolated hepatocytes and in its plasma membrane fraction was caused by equimolar amount or by 10~100 fold oversupply of $NADP^+$, respectively. Over 50% inhibition could be observed with the equimolar amount. Inhibition was increased to 80% by a tenfold or to above 90% by a 100-fold excess supply of $NADP^+$. The NADH-induced CL was inhibited also by added NAD^+ (Fig. 3). This observation is of special interest because, in contrast with triphosphopyridine nucleotides in the cytoplasm of liver cells which only 0.5~2.5% are in the oxidized state as $NADP^+$, there is a 200 to 1200-fold surplus of NAD^+ over NADH with the diphosphopyridine nucleotides in the cytoplasm of liver cells (Gumma *et al.*, 1971). I observed the inhibiton of the initial rate of NADH-induced CL by increasing amounts of NAD^+ with hepatocytes and its plasma membrane fraction by 75% (Fig.3).

NADH and NADPH induced CL, and stimulated glucose uptake are shown in Fig. 4. It could not be ruled out that they supplied the reducing equivalents to different dehydrogenases consisting the initial component of $\cdot O_2^-$-forming systems. In such a case, both pyridine nucleotides should not mutually influence each other in their action on CL. But Fig. 3 shows that the initial rate of CL induced by 20 μM NADH was inhibited even more strongly by 0.2 mM $NADP^+$ than by 0.2 mM NAD^+. It is likely, therefore, that both nucleotides have an identical access to the $\cdot O_2^-$-forming system.

In addition, 1 mM calcium ion obstructs the initial rate of CL development after addition of NADH or NADPH, but it stimulates CL in a NADPH-regenerating system (Table 1). The integrated number of photons, the total CL yield, was independent of calcium.

For this purpose, experiments of 30 or 60 sec duration were conducted and the initial rate was always taken by the initial linear regression with the slope of CL kinetics. The half maximal value is about 6 μM for NADH and 30 μM for NADPH in plasma membrane, without a NADPH-regenerating system. For hepatocytes the K_m values are 1.5 μM± 0.8 for NADH and

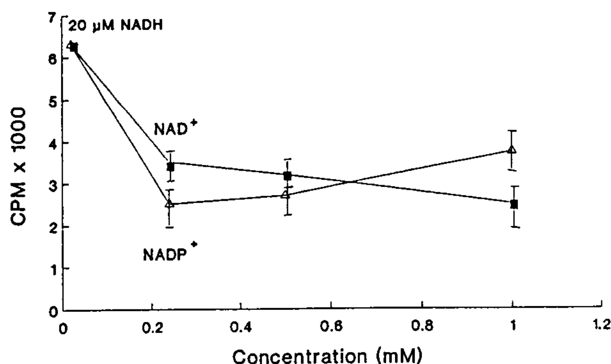

Fig. 3. Comparison of the inhibition of NADH-induced CL by various concentrations of either NAD^+ or $NADP^+$ with plasma membrane. The assay was started with 20 µM NADH. Incubation was carried out with 60 µg plasma membrane, 10 µM lucigenin and 1.5 ml incubation medium. The preincubated concentrations of NAD^+ or $NADP^+$ were 0.25, 0.5 and 1 mM.

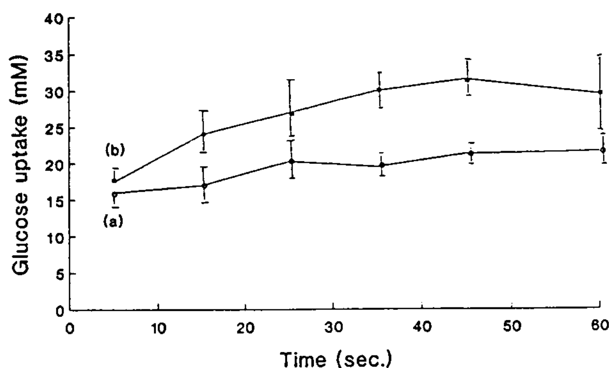

Fig. 4. Effect on glucose uptake of hepatocytes by NADH. 16 mM glucose and ^{14}C-D-glucose were added in (a) 20 µM NADH and ^{14}C-D-glucose were added at 2 min before 16 mM glucose in (b) Incubation was carried out with 1 mg hepatocytes protein/ml and incubation medium at 37℃ as described under "Materials and Methods".

55 µM± 7.4 for NADPH in the presence of a NADPH-regenerating system. K_m values are obtained from the half maximal concentrations in the saturation curve by taking highest CPM (counts per minute). In the case of the NADPH-regenerating system, NADPH was added, instead of $NADP^+$ as is usually done in measurements of cytochrome P_{450}-activity. Because I found that with high $NADP^+$ concentrations in the mM range, 30 min preincubation at 37℃ is not sufficient for complete reduction of $NADP^+$, so that $NADP^+$-induced decrease of CL can not be fully prevented. Accordingly the initial velocity was decreased if $NADP^+$ was added at higher concentrations (Kim, 1993).

If NADH is given again after all the NADH was consumed, characteristic profile of CL was changed. Renewed NADH addition increases the maximum value and the integrated total amount of counts.

The generation of CL by phagocyte has been shown to be a valuable tool for monitoring their phagocytic activity. It has been used to investigate mechanisms by which stimulants or inhibitors affect the function of phagocytic cells. For this reason, CL has been used as assay system to detect various disease states (Trusch *et al.*, 1978).

Both nucleotides, NADH and NADPH generate CL not only in plasma membrane fractions but also with intact hepatocytes. Contrary to hepatocytes, induction of lucigenin-CL of plasma membrane requires addition of reduced pyridine nucleotide. Pyridine nucleotide-increased CL in hepatocytes adds to a basal CL controlled by cellular factors. The characteristics of CL produced by either nucleotide are differnt. Contrary to plasma membrane, the CL of hepatocytes increases over a longer period of time. The decrease of NADPH-induced CL occurs later.

Table 1. Effect of calcium on lucigenin-CL induced by addition of either 20 µM NADH, 20 M NADPH or a NADPH-regenerating system to hepatocytes. Incubation was carried out with 1.5 ml incubation medium, hepatocytes-protein 1 mg/ml at 37℃. 20 µM $NADP^+$, 13.3 mM isocitrate and 133 µg/ml isocitrate dehydrogenase were added in (c)

		Initial CPM×10^3	CL-integrated counts×10^6
NADH (a)	1 mM Ca^{2+}	24.5	14.8
	No Ca^{2+}	49.1	14.7
NADPH (b)	1 mM Ca^{2+}	61.3	8.2
	No Ca^{2+}	76.9	7.3
NADPH-regenerating system (c)	1 mM Ca^{2+}	124.5	15.9
	No Ca^{2+}	98.2	16.7

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