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Production of hydrogen peroxide in rat corpus cavernosum: An on-line study with microvoltammetric electrodes

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Abstract

Continuous measurements were realized in rat corpus cavernosum using microvoltammetric captor. After inhibition of endothelial NO-synthase by L-NMA vasodilatation was induced by intracavernous injection of acetylcholine. Blood flow of rat penis was monitored by laser Doppler. A new peak appeared in reduction. It was identified as hydrogen peroxide by its peak potential, by intracavernous injection of hydrogen peroxide or catalase. Intracavernous injection of various pharmacological agents permitted to demonstrate that its origin was due to endothelial NAD(P)H oxidases. DPI inhibited its synthesis; NADH and NADPH enhanced it. Intracavernous injection of diethyldithiocarbamic acid gave the disappearance of hydrogen peroxide peak and appearance of the superoxide peak. This preliminary study showed that when the L-arginine pathway was inhibited, the NAD(P)H oxidase pathway functioned in rat corpus cavernosum for vasodilatation. ***To cite this article: A. Meulemans, C. R. Biologies 328 (2005).***

Résumé

Des mesures en continu ont été réalisées dans le corps caverneux du rat à l'aide d'un microcapteur voltamétrique. Après inhibition de la NO-synthétase endothéliale par du L-NMA, la vasodilatation fut induite par injection ic d'acetylcholine. Le flux sanguin pénien du rat fut suivi par LASER Doppler. Un nouveau pic est apparu en réduction. Il a été identifié comme étant de l'eau oxygénée par le potentiel de son pic, par injection intracaverneuse d'eau oxygénée et de catalase. L'injection intracaverneuse de différents agents pharmacologiques permet d'affirmer que son origine est due aux NAD(P)H oxidases endothéliales. L'injection intracaverneuse de DPI inhibait sa synthèse et celles de NADH et NADPH l'augmentait. L'injection intracaverneuse d'acide diéthyldithiocarbamique entraîne sa disparition, avec apparition du pic du superoxyde. Ce travail préliminaire montre qu'une fois le système de la L-arginine inhibé, le système des NAD(P)H oxidases le remplace dans le corps caverneux du rat pour la vasodilatation. ***Pour citer cet article : A. Meulemans, C. R. Biologies 328 (2005).***

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Mots-clés : Eau oxygénée ; Voltamétrie ; Superoxyde ; FHDE ; Pénis

1. Introduction

Recently voltammetry was used in the reduction mode to study the production of NA (nitroso-arginine), a product of endothelial NO-synthase by the endothelial cells of rat corpus cavernosum [1]. It was observed that the L-arginine pathway was used during rat penis vasodilatation in normal physiological conditions. Authors [2] reported that rat corpus cavernosum endothelium used exclusively in vivo the endothelium derived relaxing factor (EDRF) derived from the L-arginine pathway. They discovered that the cGMP pathway was the only system to mediate the penile erection in rats [2]. In rat other vascular beds, the most important relaxing factors come either from the L-arginine pathway, from prostacyclin pathway or from reactive oxygen species (ROS) pathway [3,4]. A remaining question was to know the nature of the mediator of vasodilatation in rat corpus cavernosum when the L-arginine pathway was inhibited. As soon as 1988, Taylor and Weston proposed an additional factor to EDRF which was called endothelial derived hyperpolarizing factor (EDHF) [5]. EDHF caused relaxation with simultaneous increase of the membrane potential, which differentiated it from EDRF. EDHF was described as an endothelium-derived non-nitric oxide (NO) and non-prostacyclin factor that mediated hyperpolarization of vascular smooth muscle via calcium-sensitive K^+ channels aperture. Considerable works have been done to identify this factor and its true nature was still matter of debate, as seen in literature and in the recent congress of Antwerp [6]. Its nature seemed to be dependent of the vascular bed. EDHF might have a predominant role in small resistance arterioles in rats. At this time there was no information on the nature of EDHF in rat corpus cavernosum. In other species like mice, the cAMP pathway played a role in corpus cavernosum [7] and hydrogen peroxide was described in mice corpus cavernosum as an endothelium-derived relaxing factor (EDRF) and hyperpolarizing factor (EDHF) using in vitro experiments [8]. The source of hydrogen peroxide was proposed as either eNOS or NAD(P)H oxidases. The two enzymes were present in endothelial cells. NAD(P)H oxidases were present in endothelial cell membranes. It secreted superoxide ($O_2^{\bullet -}$) [9]. Superoxide dismutase (SOD) transformed $O_2^{\bullet -}$ into H_2O_2 in the extracellular space. H_2O_2 was a mild oxidant using the cGMP pathway. Then H_2O_2 could be a serious candidate to vasodilatation in rat penis. Prostacyclins did not use the cGMP pathway and were not candidates for vasodilatation in rat penis. Studies showed a cross-talk between EDRF and EDHF in the regulation of endothelium mediating vasorelaxation [10]. The inhibition of EDRF in rat corpus cavernosum could reveal EDHF if present and detectable by voltammetry. As candidate, H_2O_2 was described as electroactive either in oxidation or in reduction. But in oxidation H_2O_2 peak potential was located near catecholamine, ascorbate or NO peaks depending of the different microelectrodes [11–13]. ROS were generally electroactive, but it is quite difficult to identify and quantify generated free radicals in vascular tissues at the same time. Amperometric techniques at fixed potential have been developed for $O_2^{\bullet -}$ and H_2O_2 with different microelectrodes [14–17]. But their selectivity for ROS in vivo is a major problem due to a fixed potential and to very low current measurements (a few pA).

Recently, reductive differential pulse voltammetry (RDPV) showed the ability to detect $O_2^{\bullet -}$, H_2O_2 and NA using the electroactive domain in reduction that was not used with microelectrodes by others, excepted for oxygen measurements on platinum in vivo [18, 19]. Here the oxygen interferences were absent, due to the carbon surface of the microelectrode, which reacted slowly with oxygen and gave a poor detection limit. RDPV gave simultaneous identification of a compound by its peak potential and quantification by the height of this peak. The sensitivity limit was the µM range with currents in nA range. The advantage of RDPV was the absence in vivo of endogen peaks, excepted NA peak if present. This is not the case in the oxidative domain where many endogen molecules are electroactive in vivo.

The present study outlines RDPV in vascular endothelium of corpus cavernosum of living rats us-

ing a micro carbon electrode during simultaneous vasodilatation by acetylcholine and inhibition of the L-arginine pathway by L-NMA. The identification of new endogen RDPV peaks was realized either in vitro by their peak potential values or in vivo by intracavernous injection of pharmacological agents acting on these peaks presence, apparition, increase and disappearance.

2. Material and methods

2.1. Materials

Acetylcholine (Ach), L-NMA, L-arginine, bradykinin, arachidonic acid, indometacin, reduced nicotinamide dinucleotide (NADH), reduced nicotinamide dinucleotide phosphate (NADPH), diethyldithiocarbamate (DDTC), catalase and SOD were purchased from Sigma (Saint-Quentin-Fallavier, France). H_2O_2 was from Merck (Noisy, France). Diphenyl-iodonium (DPI) was from Calbiochem (France).

2.2. Voltammetry

A microelectrode was built using a microcarbon fibre (8–12-µM diameter, Le Carbone Lorraine, France) inserted in a glass microelectrode and stretched in a microforge according to previous works [20]. It was commercially available (MFC1, Tacussel, Lyon). The reference and auxiliary electrodes were respectively Ag/AgCl and platinum. All measurements were performed with three electrode systems: one apparatus (PRG5, Tacussel, France), which was automated with relay cards and AD/DA cards, inserted in a microcomputer or an integrated apparatus (Autolab, PGSTAT 10, Roucaire, France). These two apparatus gave identical results. RDPV was performed with the following parameters: pulse height = 0.1 V, pulse delay = 0.1 s, potential swept = 0.04 V s^{-1}. The starting potential was −0.5 V vs. Ag/AgCl. The swept were made in reduction. No Faraday cage was needed for measurements according to nA current range. Standardizations for H_2O_2 was realized in vitro in phosphate buffered saline (PBS) at 20 °C at oxygen atmospheric pressure at −1.02 V vs. Ag/AgCl (range 1–200 µM). With these microelectrodes, the sensitivity limit was µM for H_2O_2. Other compounds can be detected in reduction. For NO the limit of detection was µM at −1.37 V vs. Ag/AgCl. Superoxide was also detected at µM at −0.79 V vs. Ag/AgCl. Oxygen was undetectable under 1 mM at −0.67 V vs. Ag/AgCl. The observed signal in vivo for RDPV was the NA peak at −1.66 V vs. Ag/AcCl, as previously described [1].

2.3. Animals

Male adult rats (Sprague Dawley) were 432–544 g (Janvier, Le Genest-Saint-Isle, France). They were anesthetized with urethane (1.45 g kg^{-1}, ip) and were on a homemade heating table during measurements. The penis was exposed and a small incision (1–2 mm) was realized. Then the voltammetric microelectrode and a micro glass-device for intracavernous injection of pharmacological agents were introduced with micromanipulators (Prior, Phymep, France) in the corpus cavernosum near the voltammetric microelectrode under binocular observation (Zeiss, France). Each drug was dissolved in PBS at 1 mM (37 °C); 40–50 µl were injected each time. DPI was dissolved in dimethylsulphoxide (DMSO). Intracavernosal injection of a compound was made at least in three different animals. Vasodilatation of penis was monitored using laser Doppler flowmetry. Briefly, a microprobe was put on the penis near the microelectrode (PF418 master probes and Periflux 4001 master, Perimed, France). It gave on line the blood flow in flow units. In a first series ($n = 10$ rats) RDPV was realised every minute with simultaneous laser Doppler flowmetry after intracavernous injection of acetylcholine (50 µl, 100 µM); Then 30 min later, a mixture of acetylcholine plus L-NMA was injected ic (50 µl Ach 100 µM, L-NMA 1 mM). In a second series ($n = 36$ rats), RDPV was realised every minutes after intracavernous injection of the same mixture of acetylcholine and L-NMA; then at 30 min a compound ($n = 12$) was injected ic. In each series RDPV was monitored 2 h in vivo. Each rat received one compound and this compound was tested on three rats.

3. Results

In the first group, NA peak was present after acetylcholine injection as previously described [1]. The simultaneous injection of acetylcholine and L-NMA gave the disappearance of NA peak with appearance of a new peak at −1.02 V vs. Ag/AgCl (see Fig. 1). This

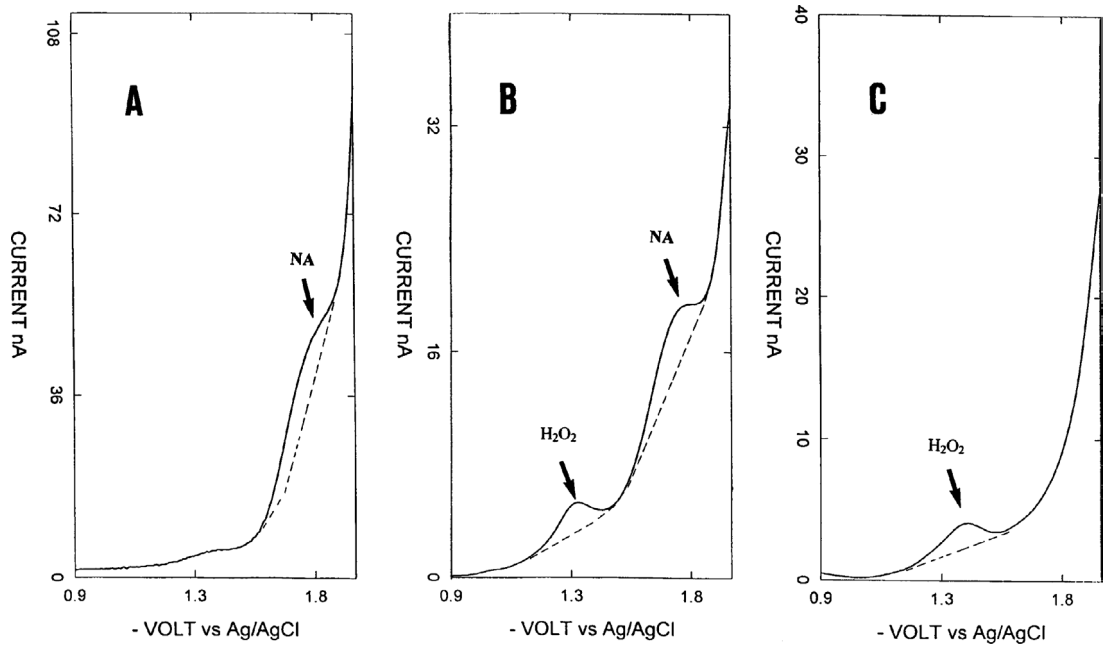

Fig. 1. An example of voltammetric signals in a rat corpus cavernosum. (**A**) Basal signal after ic injection of acetylcholine. (**B**) Signal 1 min after ic injection of a mixture of acetylcholine and L-NMA. (**C**) Signal 5 min after ic injection of a mixture of acetylcholine and L-NMA.

new peak was stable during the experiment. Its peak potential is identical to the peak value of H_2O_2. According to standardization, H_2O_2 concentration was 0.115 ± 0.055 mM ($n = 10$) in vivo. Laser Doppler flowmetry gave blood flow units multiplied by 10.9 compared with the basal value at 6 min after ic injection of Ach and L-NMA. The voltammetric peak height and blood flow units were correlated during measurements ($r = 0.998$, $n = 150$, measuring time: 0–15 min after ic, $n = 10$ rats).

In the second group, the identity of the peak and the biological system responsible were studied. Results are summarized in Table 1. Briefly, in vivo injection of H_2O_2 near the microelectrode tip in corpus cavernosum enhanced dramatically this peak. Injection of catalase made the peak disappeared with appearance of NA peak (see Fig. 2A). This peak at -1.66 V was attributed to NA, a product of eNOS, according to previous studies [1,21–23]. SOD had no effect on the H_2O_2 peak. L-Arginine made the H_2O_2 peak disappear and the NA peak come again. DDTC injection made this H_2O_2 peak disappear and a new peak at -0.79 V vs. Ag/AgCl appeared (see Fig. 2B). This potential value corresponded to $O_2^{\bullet -}$ as previously demonstrated in vitro [19]. NAD(P)H increased H_2O_2 peak. DPI made the H_2O_2 peak disappearance. Two vasodilatators in penis bradykinin and acetylcholine enhanced the H_2O_2 peak when eNOS was inhibited. Indometacin, a cyclo-oxygenase inhibitor and arachidonic a prostanoid precursor had no effect on the H_2O_2 peak.

4. Discussion

4.1. Identification of the endogen peak

RDPV was realized on line in rat corpus cavernosum. The microelectrode tip was located in the extra vascular space in contact with the endothelial cells of the sinus. The microelectrode detected compounds at the contact of its active tip. When the microelectrode tip was removed of the cell surface contact the signal immediately disappeared. Measurements corresponded to punctual concentrations at the interface of the microelectrode tip and endothelial cells membranes. A substance was detected when its concentration was superior to the sensitivity limit of RDPV for this molecule. Before penis-induced vasodilatation, no signals were observed in RDPV, indicating

Table 1
Effect of intracavernosal injection of various compounds on hydrogen peroxide concentrations

Various compounds	H_2O_2 concentration (% initial value). Time 5 min after ic	Half-life of H_2O_2 disappearance or apparition (sec) after ic	Nature of other peak after H_2O_2 disappearance
Catalase	0	12	NA
SOD	unchanged	zero	no other peaks
L-Arginine	23	244	NA
H_2O_2	256	123	no other peaks
NADPH	154	487	no other peaks
NAD	141	539	no other peaks
DDTC	0	58	superoxide
Acetylcholine	137	282	no other peaks
DPI	8	185	no other peaks
Bradykinin	146	220	no other peaks
Indometacin	unchanged	zero	no other peaks
Arachidonic acid	unchanged	zero	no other peaks

Each value was the mean of 3 rats.

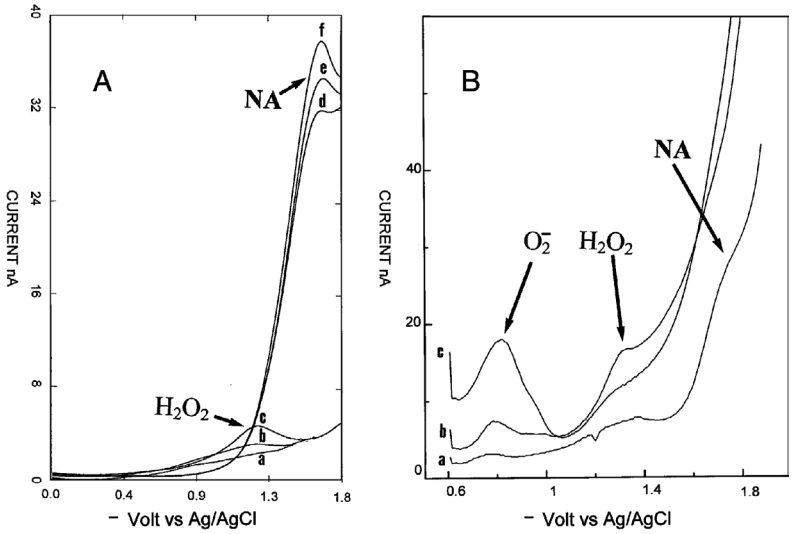

Fig. 2. An example of variation of hydrogen peroxide in a rat corpus cavernosum. (**A**) Voltammetric signal after injection of acetylcholine and L-NMA: (**a**) before injection, (**b**) 2 min, (**c**) 5 min. Then injection ic of catalase: (**d**) 1 min, (**e**) 2 min, and (**f**) 3 min after injection. (**B**) Simultaneous injection ic of acetylcholine, L-NMA and DDTC: (**a**) before injection, (**b**) 1 min, (**c**) 2 min after injection.

either an absence of electroactive compounds or an insufficient concentration of electroactive products. This is highly favourable for the study of vasodilatation by voltammetry. Simultaneous injection of acetylcholine and L-NMA produced vasodilatation followed by the laser Doppler apparatus and appearance of a new peak. Its precise potential localization permitted to attribute this signal to H_2O_2. It was observed that molecules had the same peak potential either in vitro or in vivo. According to the fact that only one endogen molecule, NA, have been observed in vivo as electroactive in reduction on carbon microelectrode [18–20], the identification of H_2O_2 is quasi certain. Injection of H_2O_2 near the microelectrode tip confirmed this identity. The fact that injected H_2O_2 induced vasodilatation was another important point. The third proof was found by the injection of catalase, which was specific for H_2O_2. Catalase gave an immediate disappearance of the endogenous peak. Microelectrodes were easily standardized in vitro for H_2O_2 in PBS without oxygen interferences according to an endogen level below the sensitivity limit. The observed concentrations in

rat corpus cavernosum (0.115 μM) during vasodilatation were close to the concentrations observed in rat brain for H_2O_2 using microdialysis [24,25]. The stability, the repeatability, and the high current values of these peaks with always the same potential permitted to follow and quantify H_2O_2 concentrations in the extracellular space without any problems.

4.2. Biological origin of H_2O_2

The origin of H_2O_2 was studied by injection of pharmacological products. H_2O_2 production in vivo was well known and different enzymatic origins could be responsible of its secretion. Xanthine oxidase, eNOS, P450 enzymes and NAD(P)H oxidases were candidates to synthesize H_2O_2 in vivo in various cells. In endothelial cells eNOS and NAD(P)H oxidases have been described to produce H_2O_2. Precedent work on eNOS demonstrated that $O_2^{\bullet -}$ was not produced at a detectable level by voltammetry in pure enzymatic preparation, even in the absence of the substrate. The enzyme, eNOS, was not the source of extracellular release of H_2O_2 in this system, as observed in endothelial cells [1,7]. Intracavernous injection of DDTC, a SOD inhibitor, gave H_2O_2 peak disappearance along with the appearance of a new peak. This new peak corresponded to $O_2^{\bullet -}$, which was identified by its peak potential. This was the first time that $O_2^{\bullet -}$ was observed in vivo by RDPV. This was due to the high concentration value of H_2O_2 in vivo according to the equimolar transformation of $O_2^{\bullet -}$ into H_2O_2 by SOD. SOD injection had no effect on H_2O_2 peak in vivo. These facts could be explained by the fact that $O_2^{\bullet -}$ was produced inside the cells and that H_2O_2 was only present in the extracellular fluid where is located the microelectrode [26]. Another point was the presence of extracellular SOD, which immediately scavenged $O_2^{\bullet -}$ to H_2O_2 without needing an exogenous source of SOD. DDTC experiments showed that $O_2^{\bullet -}$ was the source of H_2O_2. DDTC can penetrate into endothelial cells. It is not the case for injected SOD. Substrates and inhibitor of NAD(P)H oxidases were tested. DPI, a specific NAD(P)H oxidases inhibitor, inhibited immediately this production. This indicated an enzymatic synthesis for H_2O_2 by NAD(P)H oxidases. NADH and NADPH injections enhanced the H_2O_2 peak, indicating NAD(P)H oxidases system for H_2O_2 production. The vasodilatation measured by laser Doppler flowmetry supported the idea that H_2O_2 was a vasodilatator in the corpus cavernosum bed. This was observed in other rat vascular tissues. H_2O_2 was found in pulmonary arterial walls where it is secreted by endothelial cells using NAD(P)H oxidases localized in the cells membrane [27,28]. It was observed in calf or bovine pulmonary arteries a cyclic guanosine monophosphate (cGMP)-mediated relaxation occurring through an increased intracellular H_2O_2 generation. It was also observed in rat aorta [29]. NA was not the sole agent of penile vasodilatation, H_2O_2 is another mediator of vasodilatation using cGMP pathway. Two molecules of the prostanoid system were tested. Indometacin and arachidonic acid had no effects on the H_2O_2 peak, eliminating the prostanoid pathway for its production.

4.3. Proposed mechanism of vasodilatation in rat penis

In normal physiological conditions the L-arginine pathway was the sole used for vasodilatation in rat corpus cavernosum. When this system was blocked, another system using endothelial NAD(P)H oxidases was used with production of H_2O_2, a potent physiological vasodilatator. Intracavernous injection of L-arginine made H_2O_2 disappear with simultaneous apparition of NA, the product of eNOS. The presence of L-arginine imposed the synthesis of NA. NA is the mediator of vasodilatation in normal conditions [1]. There was a switch mechanism between the two systems depending of the availability of eNOS to synthesize NA. An absence of L-arginine or the inhibition of eNOS switched the eNOS system to the NAD(P)H oxidases system. The ability of voltammetry to monitor NA, H_2O_2 and $O_2^{\bullet -}$ was very useful for continuous on line measurements in tissue. In mice, H_2O_2 was presented as EDHF and it could be the case in rat corpus cavernosum. NA was presented as EDRF in the same tissue; if true, voltammetry could monitor simultaneously EDRF and EDHF in a living animal. Rat corpus cavernosum was a very useful model for such studies.

5. Conclusion

In conclusion, this study showed on-line in vivo determination and identification of H_2O_2. Voltammetry

demonstrated that $O_2^{\bullet -}$ was the primary source of endogenously produced vasoactive H_2O_2. The NAD(P)H oxidases endothelial system was the source of this endogen vasodilatator, which replaced the eNOS system when it was unable to function normally.

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