Antioxidative Properties of Pig Vesical Mucosa: A Comparison with Gastric and Intestinal Mucosa

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The antioxidative properties of pig urinary bladder mucosa were compared with those of gastric and intestinal mucosae using nitroxide radicals. Electron paramagnetic resonance (EPR) method was used to monitor the metabolic processes of nitroxides in mucosae. The reduction of nitroxides was measured on intact luminal surfaces of gastric, intestinal, and urinary bladder mucosa, as well as in homogenates of mucosae surface layer. Furthermore, N-ethylmaleimide and ascorbate oxidase have been used to characterize the reducing agents in urinary bladder mucosa homogenates. The nitroxide concentration decrease on intact mucosa of the urinary bladder was significantly different from those of the gastric and the intestinal mucosa. The concentration decrease was the largest for intestinal mucosa and the smallest for bladder mucosa. On the other hand, homogenates exhibit the largest nitroxide reduction rates for the bladder mucosa and the smallest for the gastric mucosae. In the bladder surface layer homogenates ascorbate and thiol-containing reducing agents were found and their coupled action in the nitroxide reduction process was established. The mucosa of urinary bladder is protected against nitroxide free radicals by a relatively low permeability and very active endogenous reducing agents. The gastric and intestinal mucosae are more permeable and/or have greater antioxidant activity on their surface. The reduction of nitroxides in the urinary bladder mucosa occurs via the ascorbate–thiol coupled reducing system.

Key words pig urinary bladder; mucosa; nitroxide radical; antioxidant

The antioxidative properties of biological systems are frequently studied using a non-destructive spectroscopic method, electron paramagnetic resonance (EPR), and nitroxides as models of biological free radicals. Nitroxides are stable radicals—paramagnetic substances—which living systems metabolically transform into the non-paramagnetic substances (EPR silent), hydroxylamines. The main metabolic transformation of nitroxides is the reduction by ascorbic acid and thiol reducing agents, which can be mediated by enzymatic mechanisms involving also mitochondria and microsomes. Therefore, the kinetics of nitroxides metabolism provides useful information about the antioxidative properties of the investigated system.

The antioxidative properties of gastrointestinal mucosa are intensively studied and regional differences in the stomach, differences along the gastrointestinal tract, and also species and gender related differences have been established. On the other hand, the studies of antioxidative properties of urogenital tract are very scarce. Therefore, the aim of our study is to evaluate the antioxidative properties of pig urinary bladder mucosa and compare them with those of gastric and intestinal mucosa using three different nitroxides and EPR spectroscopy. Our work focuses also on the types of antioxidants present in the urinary bladder mucosa.

MATERIALS AND METHODS

Nitroxides We used water soluble nitroxides-spin probes, namely: ASL [N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-N-dimethyl-N-hydroxyethylammonium iodide], which has a permanently positive charge in water solutions, GluSL [4-(2,3,4,5,6-pentahydroxyhexanoylamino)-2,2,6,6-tetramethylpiperidinyl-1-oxyl], which is uncharged in water solutions, and PCA [2,2,5,5-tetramethylpyrroline-1-oxyl-3-carboxyl acid], which is partially ionized and therefore negatively charged in water solutions. The chemical structures of nitroxide compounds used are shown in Fig. 1. The nitroxides ASL, PCA, and GluSL were synthesized according to procedures published elsewhere.

Tissue Preparation Stomachs, small intestines, and urinary bladders from freshly slaughtered 7 month-old pigs of both sexes, weighing 90—110 kg, were obtained from a local slaughterhouse. A small segment of the cardiac region of the stomach, a 20 cm long part of the jejunum (1 m distant from the stomach), and the urinary bladder were first gently washed and then kept in ice-cold and carbogen (95% O2, 5% CO2) saturated phosphate-buffered saline (PBS) (Ph.Eur. 3rd) pH 7.4 until the beginning of the experiment. The experiments were performed within a period of 1 to 3 h after the animals were slaughtered on each isolated tissue only once.

Fig. 1. Chemical Structures of the Nitroxide Spin Probes ASL, GluSL and PCA

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Therefore, the number of measurements is equivalent to the number of animals used in the experiments.

**EPR Measurements** The EPR measurements were performed on a Bruker ESP 300 X band spectrometer at room temperature. Typical spectrometer settings were: center field 342 mT, microwave frequency 9.6 GHz, modulation amplitude 0.1 mT, sweep 10 mT, receiver gain $5 \times 10^4 - 3.2 \times 10^4$, sweep time 84 s, time constant 40.96 ms, and microwave power 10 mW. The nitroxide metabolism was monitored by the nitroxide reduction rate via the signal amplitude of the first line in the EPR spectrum, which was proportional to the nitroxide concentration.

**The Metabolism of the Nitroxides on the Intact Surface of Mucosa, Experiment I** Circular segments of the examined tissues (the muscularis externa of the stomach was removed) were mounted in the diffusion chamber (Fig. 2) so that only the mucosal surface of the tissue (4.5 cm$^2$) was exposed to the nitroxide PBS solution, while the serosal surface was isolated. The mucosa was first exposed to the applied 5 ml of PBS solution, and subsequently, an appropriate volume of nitroxide PBS solution was added. The system was exposed to the air, the solution was constantly stirred, and every five minutes a sample of the solution was drawn into a glass capillary for EPR measurements, with a 1 mm inner diameter. The first measurement was performed 30 s after addition of nitroxide to PBS.

**The Metabolism of the Nitroxides in the Surface Layer Homogenates, Experiment II** The luminal surface of gastric, intestinal or bladder mucosa was gently scraped with a plastic card. The gastric, intestinal, and bladder-scraped mucosal area needed for obtaining the 500 $\mu$l of the surface layer homogenates, ranged from 5—7.5 cm$^2$, 10—15 cm$^2$, and 70—110 cm$^2$, respectively. The obtained samples were homogenized with the Potter-Elvehjem homogenizer or vigorously stirred on vortex (the results obtained by both methods did not differ significantly). To 50 $\mu$l of the surface layer homogenate an appropriate volume of nitroxide PBS solution was added to achieve the defined concentration. The final volume of the mixture was adjusted to 100 $\mu$l with PBS. After the addition of nitroxide, the mixture was stirred on vortex for 45 s and drawn into a glass capillary for EPR measurements. The time interval between the addition of nitroxide and the beginning of EPR measurements was 3 min. The measurements in the capillary were performed at various time intervals. As the initial reduction of nitroxide in surface layer homogenates appeared to follow first order kinetics, the zero time signal amplitudes in the experiments II and III were obtained by curve extrapolation.

In order to evaluate the number of cells in homogenates, 50 $\mu$l of the scraped sample was appropriately diluted with PBS. 50 $\mu$l of the resulting mixture was combined with 1% solution of Trypan blue (Merck, Germany) in PBS at a ratio of 1:1. The cells' nuclei were counted using a counting chamber with defined volume (Bürker-Türk, Fein-Optik, Germany) and a phase contrast microscope (Olympus BX50, Japan).

**Determination of Antioxidants Present in the Bladder Surface Layer Homogenates, Experiment III** The involvement of thiol agents and ascorbic acid in metabolic processes of nitroxides in the bladder surface layer homogenates was determined with N-ethylmaleimide (NEM, Sigma, U.S.A.) which alkylates thiol groups and ascorbate oxidase which oxidizes ascorbate to dehydroascorbate (AO, Sigma, U.S.A., one unit oxidizes 1.0 $\mu$mol of L-ascorbate per min at pH 5.6 at 25°C).

Fifty microliters of the bladder surface layer homogenate was mixed with the appropriate volume of freshly-prepared NEM solution in PBS, in order to achieve various concentrations ranging from 0.1 to 12 mm. After 30 min of incubation at room temperature, the PCA solution in PBS was added to the mixture. The final volume of the mixture was 100 $\mu$l and the concentration of PCA was 0.15 mm. The mixture was stirred on vortex for 45 s and drawn into the glass capillary for EPR measurements; 3 min after the addition of PCA, the EPR spectra were recorded at various time intervals.

The volume of bladder homogenate, the PCA addition and its final concentration, the volume of the final mixture, and the conditions of EPR measurements were the same also for the experiments described below.

For experiments where AO was used, the final concentration of AO in the mixture ranged from 0.001 to 1.3 unit/$\mu$l. Before PCA addition, the mixture was incubated at room temperature for 30 min.

In order to separate the role of thiols from ascorbate in the metabolic processes of PCA in bladder homogenates, the influence of combination of NEM and AO was compared with the influence of NEM. The homogenate was first incubated with NEM solution in PBS (final concentration in mixture was 10 mm) for 90 min, and after the addition of AO solution in PBS (final concentration in mixture was 0.3 unit/$\mu$l) for another 30 min. We also measured the metabolism of PCA only in presence of NEM under the same conditions as in above example of two agents ($c_{NEM}=10$ mm, time of incubation 120 min).

**Statistical Analysis** The results are expressed as mean values and standard deviations. The statistical significance of nitroxide metabolism in various mucosae and homogenates was assessed using the two-sample $t$-test. Before applying this test, the $F$-test was used for evaluation of homoscedasticity. Depending on the results of this test, the $t$-test for equal or unequal variances was performed. The slopes and inter-
cepts of regression lines through the logarithmic values of experimental data were used for statistical analysis. Values of $p<0.05$ were considered significant.

RESULTS

The nitroxide reaction kinetics was used to apprise the antioxidative activity of the mucosa tissue endogenous agents. Two experimental approaches were used. In the experiment I, performed on the intact mucosa, the reaction rate was controlled by the membrane permeability and antioxidants present on the surface of mucosa, while in the experiment II free access of nitroxides to the reducing agents in the homogenate provided the complementary information on the activity of antioxidants present in cells, in intercellular fluid, and in mucus. Additionally, on the example of the nitroxide PCA, we tried to determine the type of antioxidants involved in nitroxides metabolism in the bladder mucosa homogenates. The reactional coupling between tested antioxidants thiols and ascorbate was also investigated in experiment III.

The kinetics of nitroxide metabolism by the native reducing agents present in the mucosa was measured by EPR. The nitroxides were selected according to their solubility in aqueous media, their molecular structure and charge. Different authors have demonstrated that these properties in addition to molecular size do influence their molecular transport\textsuperscript{24} as well as their reaction rates\textsuperscript{14,15,24} in biological systems.

The results of the experiments I and II are shown in Figs. 3 and 4 where the concentrations of the nitroxides are plotted against time. The experiments on intact surface of mucosae and in the homogenates showed that the components of all three mucosae were able to reduce the nitroxides. Figure 3 shows the reduction kinetics of the nitroxides for three types of intact mucosa tissues. In spite of the previously mentioned differences in the molecular transport and reaction rates for the three chosen nitroxides, the reaction rate was always the largest for the intestinal mucosa and the smallest for the bladder mucosa. Slopes of regression lines obtained from experiments with bladder mucosa were statistically compared with slopes of regression lines for experiments with gastric and intestinal mucosa for a chosen nitroxide. The time interval from 0 to 60 min was used for ASL and GluSL regression line calculation while in the case of PCA the time interval from 20 to 60 min was chosen. The statistical analysis

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**Fig. 3.** The Time Course of ASL, GluSL and PCA Concentrations in the Experiment I on Intact Surface of Gastric, Intestinal, and Bladder Mucosa

The figure represents the average of nitroxides relative concentration ($I_t/I_{0.5}$: the ratio of signal amplitude in time $t$ to time 0.5 min) versus time. The initial concentrations of nitroxides ASL, GluSL and PCA in the PBS were: 0.1, 0.1, and 0.05 mM, respectively. All data points are presented as means±standard deviations of 3—4 measurements. The symbol # indicates statistical differences among declines of nitroxide concentration profiles obtained in experiments on the urinary bladder mucosa and declines of nitroxide concentration profiles obtained in experiments on the two other mucosae.

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**Fig. 4.** The Time Course of ASL, GluSL and PCA Concentrations in Surface Layer Homogenates (Sur. Lay. Hom.) of Gastric, Intestinal, and Bladder Mucosa (Experiment II)

The figure represents the average of relative concentrations ($I_t/I_0$: the ratio of signal amplitude in time $t$ to zero time) versus time. The zero time signal amplitude is taken at the addition of nitroxide to the homogenate. The initial concentrations of ASL, GluSL, and PCA in the mixtures were: 0.9, 0.5 and 0.15 mM, respectively. All data points are presented as means±standard deviations of 3—5 measurements. The symbol # indicates statistical differences among declines of nitroxide concentration profiles obtained in experiments on the urinary bladder mucosa homogenates and declines of nitroxide concentration profiles obtained in experiments in the two other mucosa homogenates.
The reduction rate was calculated from the difference of nitroxide concentrations in the third and fifth minute. The absolute values of the reduction rates are plotted against NEM concentrations. The sigmoid curve is a response of the Hill’s equation.

showed that the declines of concentration profiles of nitroxides obtained in experiments on urinary bladder mucosa were significantly different from those of gastric and intestinal mucosa (Fig. 3), rendering probability value $p<0.05$. However, in the case of GluSL, the value $p$ for the pair intestine/bladder mucosa was slightly higher than 0.05 ($p=0.072$) due to the high variability of intestine measurements. Simultaneously, the intercepts of regression lines were also statistically compared for the same pairs of experiments as in the case of statistical evaluation of regression slopes. No statistical significance was demonstrated. On the other hand, the results in Fig. 4 show that the homogenates exhibit the largest nitroxide reduction rates for bladder mucosa and the smallest for the gastric mucosa. Slopes and intercepts of regression lines for experiments with urinary bladder homogenates were compared with slopes and intercepts of regression lines for experiments with gastric and intestinal homogenates. The statistical analysis showed that slopes of regression lines for bladder homogenates significantly differ from those of gastric and intestinal homogenates for all studied nitroxides, whereas intercepts of regression lines did not differ significantly.

The number of cells’ nuclei ranged from $3 \times 10^4$ to $7 \times 10^5$ per microliter of homogenate, with the highest number of cells’ nuclei in the intestine, lower in the bladder, and the lowest in the gastric mucosa homogenates. The rank order of cells in homogenates is not the same as reduction activity of homogenates, this means that different number of cells was not the reason for different reduction properties of homogenates.

In experiment III, we tried to determine the type of reducing agents in the bladder mucosa surface layer homogenates. Therefore, the NEM was applied to alkylate SH groups of the reducients in the samples treated by PCA (Fig. 5). We used different NEM concentrations ranging from 0.1 to 12 mM in the final mixture. The results are interesting since there is a sharp separation of the PCA reduction rates depending on the concentration of the inhibitor. The concentrations which were lower than 3 mM did not affect the nitroxide reduction kinetics. Their kinetics were the same as for the control. A sharp decrease in reduction rates occurred if 4 mM or higher concentrations of NEM were used.

To test the involvement of ascorbate in the reduction processes, different concentrations of AO were added to the PCA homogenates. Linear decrease of the PCA concentration in natural logarithmic plot for the initial time interval of 17 min was observed (correlation coefficient $r>0.99$) and showed that the values of the slope of regression curves for the control, 0.001, 0.01, 0.1, 1, and 1.3 unit/$\mu$l of AO activity in the final homogenate were $0.23 \text{ min}^{-1}$ ($n=8$), $0.19 \text{ min}^{-1}$ ($n=1$), $0.20 \text{ min}^{-1}$ ($n=1$), $0.19 \text{ min}^{-1}$ ($n=1$), $0.24 \text{ min}^{-1}$ ($n=1$), and $0.25 \text{ min}^{-1}$ ($n=1$), respectively. These results allow the conclusion that no significant change in the PCA reduction kinetics occurred with increasing AO concentration. However, from the Fig. 6, where the reduction kinetics of PCA homogenate in presence of both NEM and AO is shown, the role of ascorbate in the reduction process is evident. The statistical significant differences between slopes of the regression lines referring to homogenates incubated with NEM and combination of NEM and AO were established, whereas intercepts of regression lines did not differ significantly. The results of experiment III corroborated the coupling mechanism between the thiol and ascorbate system.

**DISCUSSION**

Although nitroxides are one of the most stable radicals known, they are not chemically inert and they are metabolized in tissue. The main metabolic pathway is their reduction to hydroxylamines, which can be further reduced to amines or reoxidized to nitroxide. The mechanism of nitroxide reduction has been investigated in different biological systems. It is assumed that the reduction of nitroxides occurs via the reducing agents, which capacity is sustained by the mechanisms of the cellular metabolism. Studies of liver, skin and blood cells or homogenates have proved that ascorbate and thiols are the main agents that influence nitroxide reduction. Moreover, Fuchs suggests that analysis of
Tempo nitroxide scavenging is a convenient method for measuring skin ascorbate and thiol-dependent antioxidant activity. The reduction of nitroxide occurs primarily intracellularly,\textsuperscript{6,24} thus the ability of a nitroxide to enter the cells is one of the principal variables that affect the rate of reduction. Once they diffuse into the cells, the structure of nitroxide determines the intracellular rate of reduction.\textsuperscript{15,24} Therefore, the experimentally observed nitroxide concentration declines in tissue reflect a coupled diffusion–reduction of the nitroxide in this heterogeneous system. The structure of the chosen nitroxides and properties of the studied tissues have influenced processes also in our experiments.

During the experiment I the following parallel processes could occur: nitroxide metabolism and diffusion of nitroxide into the mucosa. The nitroxides came into contact with mucosa surface where they could be metabolized by reducers or they could diffuse into the mucosa.

The concentration profiles of PCA differ from those of ASL and GluSL (Fig. 3). For PCA, the five-membered pyrroline nitroxide, a slower nitroxide concentration decrease was observed. This was a consequence of the fact that the five-membered nitroxides undergo slower reduction than the six-membered nitroxides.\textsuperscript{15,16,24} Furthermore, the rate of hydroxylamines reoxidation, formed by reduction of aqueous soluble nitroxides, is significant only for pyrrolidine nitroxide derivatives\textsuperscript{25} which can herewith additionally attenuate the reduction rate. Therefore, in the case of PCA reduction and reoxidation rate became comparable and slower PCA concentration decrease was observed as in the case of ASL and GluSL. Moreover, a slight increase of PCA concentrations can be seen in the initial phase of experiment (Fig. 3), but the statistical analysis of the data showed that this increase could not be claimed significant. Other literature data show that the rate of penetration of PCA into the mouse thymus-bone marrow cells was relatively slow, whereas nitroxides with positive charge, like ASL, did not enter the cells.\textsuperscript{24} The nitroxide GluSL is also a very polar molecule due to its high content of hydroxyl groups. Therefore, we can presume that the penetration rate of these three nitroxides into the cells is very low.

Concerning the type of tissue, the nitroxide concentration decrease in the experiment I was the largest for intestinal mucosa, while it was the smallest for bladder mucosa (Fig. 3). It should be also stressed that the above rank of nitroxide concentration decrease was independent of the nitroxide type. These results indicate that urinary bladder mucosa is protected against free radicals by its low permeability, but on its surface it exhibits low antioxidant activity. Our results are supported by the findings of the other authors who have demonstrated that low permeability of the urinary bladder mucosa pertains to the apical membrane\textsuperscript{28,29} and tight junctions\textsuperscript{27} of epithelial superficial cells. Moreover, it seems that glycosaminoglycans, which cover the epithelial superficial cells, also represent the urinary bladder permeability barrier for small molecules.\textsuperscript{28,29} On the other hand, the intact surface of gastric and intestinal mucosa exhibit greater permeability and/or antioxidant activity.

The experiment II provided additional information on the activity of antioxidants present in cells, in intercellular fluid, and in mucus. The rank order of nitroxide concentration decrease was different as in the experiment I. The bladder homogenates had the largest and gastric homogenates the smallest reduction rates (Fig. 4). In the experiment II, equal volumes of homogenates obtained from different surface area of mucosae were used. However, in the case of the urinary bladder, the surface areas in the experiments I and II were comparable, and much higher reduction in the homogenates than on the mucosa surface could be observed. This might indicate that efficient mechanism in vivo has been developed to protect cells from free radicals. We can assume that free radicals which come in contact with mucosa probably diffuse into mucosa in very low amounts and those which enter the mucosa are efficiently eliminated by the endogenous reducing agents.

To characterize the type and the role of the reducing agents, the specific thiol alkylator NEM and ascorbate oxidase were added to bladder mucosa homogenates (experiment III). The role of thiol reducing agents was determined by varying NEM concentration and time of incubation. The reduction of PCA was significantly diminished if 4 mM or higher concentrations of NEM were used (Fig. 5). The reduction of nitroxide is obviously coupled with thiol reducing agents and only when the majority of thiol reducing agents are inhibited, the reduction kinetics of nitroxide is decreased. The possible thiol reducing agents are glutathione, mucins, different kind of enzymes, and other thiol-containing substances.\textsuperscript{30–33} The involvement of ascorbic acid in the reduction process could not be directly observed. Its role in the reduction process was determined when samples were previously incubated with NEM (Fig. 6).

These phenomena can be explained with the ascorbate-thiol coupled reducing system.\textsuperscript{34} The nitroxides are reduced by ascorbate, which is consequently oxidized to dehydroascorbate. On the other hand thiols like glutathione are not capable to reduce nitroxide directly, but they can reduce dehydroascorbate back to ascorbate. The reduction of dehydroascorbate by thiols sustains the flow of the electrons to the nitroxides and thus enables their reduction. The AO strongly increases the oxidation rate of ascorbate in the presence of oxygen. However, the measurements were performed in the glass capillaries where the amount of oxygen is limited and consumed by the metabolic oxidative processes. Therefore, the oxidation rate of ascorbate by AO via oxygen was too small in comparison with the rate of ascorbate recycling by thiols and the reduction rate of nitroxide remained unchanged. It is only when AO was applied subsequently to NEM, the effect of thiols was decoupled and the inhibitory effect of AO revealed the ascorbate presence in the homogenate (Fig. 6). However, it is also possible that enzymatic systems,\textsuperscript{17} which are directly or indirectly involved in the nitroxide reduction and can be inhibited with NEM, masked the effect of AO. Couet and coworkers\textsuperscript{14} have demonstrated the same effects of NEM, AO and combination of NEM and AO on reduction kinetics of TES nitroxide [4-(2-carboxyethylamido)-2,2,6,6-tetramethylpiperidine-1-oxyl] in a rat liver homogenate. Additionally, Mehlhorn\textsuperscript{12} has suggested another mechanism to explain the reduction process observed in erythrocytes: the formation of the putative dehydroascorbate-thiol complexes, which could be very active nitroxide reducing agents. It is possible that these complexes are also involved in our systems.

In summary, the differences among gastric, intestinal, and
urinary bladder mucosa have been demonstrated in terms of antioxidant protection against nitroxide radicals. The mucosa of the urinary bladder is protected against free radicals by a relatively low permeability and very active endogenous reducing agents. The gastric and intestinal mucosa are more permeable and/or have greater antioxidant activity on their surface. Thiols and ascorbate are important reducing agents in the urinary bladder mucosa. They are coupled into the system where thiols serve for ascorbate recycling.

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