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Effect of calcium oxalate on renal cells as revealed by real-time measurement of extracellular oxidative burst

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ABSTRACT

Calcium oxalate is one of the main constituents of kidney stones and has a proved deleterious effect on renal cells that is mediated by oxidative stress. However, the subcellular source of this oxidative stress, and whether it is extending to the extracellular space or not, is still disputed. Therefore, an electrochemical superoxide biosensor was constructed, positioned above A6 renal cells, and used to measure in real-time the extracellular oxidative burst following addition of calcium oxalate crystals. It was observed that A6 cells do secrete superoxide into their extracellular space in few minutes after encountering calcium oxalate crystals. The amount of released superoxide peaks at about 20 min. Superoxide is cleared away from the extracellular space after approximately 3 h. Superoxide secretion depends on the presence of superoxide-scavenging enzyme superoxide dismutase, the age of the cells, the amount of calcium oxalate crystals, and the temperature. Moreover, the effect of calcium oxalate crystals was mimicked by phorbol 12-myristate 13-acetate. The developed sensing system can be a useful tool for biologists investigating nephrolithiasis at cellular level.

1. Introduction

With an incidence of 0.4–1% and a prevalence of 10–12%, nephrolithiasis (i.e. calculi in the kidneys) is a common disorder that has a substantial economic impact (through the costs associated to diagnosis, emergency and surgical management as well as medical prevention) (Lotan et al., 2005). The biochemical background of nephrolithiasis is thus the subject of a very intense research. Calcium oxalate is one of the major components of renal calculi and has a proved deleterious effect on renal cells. Details of this effect are continuously revealed using analytical methods of very different complexity [such as atomic force microscopy (Rabinovich et al., 2008), cDNA macroarrays (Miyazawa et al., 2009), and two-dimensional gel electrophoresis separation combined with MALDI-Q-TOF MS/MS analysis (Thongboonkerd et al., 2008)]. Colorimetric (Khaskhali et al., 2009), fluorescent (Habibzadegah-Tari et al., 2006), or chemiluminescent (Patel et al., 2006) methods were also used. They were very useful proving that calcium oxalate, once brought into contact with renal cells, perturbs the equilibrium between the production and consumption of reactive oxygen species (ROS) leading to oxidative stress. However, fluorescent and chemiluminescent dyes for monitoring oxidative stress are loaded into the cells and are inherently acting as scavengers of ROS. They are thus not appropriate for continuous monitoring, and are always affecting the studied system to some extent. Their selectivity is questioned (Lu et al., 2006), and their sensitivity to environmental factors is also a recognized fact (Chen et al., 2008a). Most of the above mentioned analytical methods give results only after lengthy incubations of the cells with calcium oxalate, and several sample preparation steps. They are thus reporting on the long-term effects of calcium oxalate.

The lack of appropriate analytical methods has left several questions regarding the effect of calcium oxalate on renal cells unanswered. How fast renal cells show signs of oxidative stress, once brought into contact with calcium oxalate crystals, is one of them. It is still debated whether superoxide is released into the extracellular space of renal cells incubated with calcium oxalate. Some papers bring arguments for an exclusive intracellular overproduction of superoxide in cells exposed to calcium oxalate (Khand et al., 2002; Patel et al., 2006). Others claim that cell injury induced by calcium oxalate is not mediated by superoxide production at all (Borges et al., 2008).

To shed light into these issues, an electrochemical cytochrome *c*-based superoxide biosensor was constructed. Electrochemical (bio)sensors are increasingly used to answer questions regarding how biological systems function (Amatore et al., 2008; Bitziou et al., 2008; Ciobanu et al., 2008; Kita and Wightman, 2008; Lee and Kim, 2007). Encouraged by such a development, we have selected a simple superoxide biosensor that was already proved (1) to have minimal interferences from H_2O_2 (Chen et al., 2008b; Manning et

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al., 1998; Shleev et al., 2006), ascorbic acid (Manning et al., 1998), and uric acid (Ge and Lisdat, 2002; Shleev et al., 2006), (2) to withstand even the harsh experimental conditions of *in vivo* experiments (Buettemeyer et al., 2002, 2003), and (3) to have sufficient sensitivity to observe the small amounts of superoxide produced by cells (Chang et al., 2005; Manning et al., 1998; Shleev et al., 2006, 2008). A measuring chamber was also developed to accommodate the biosensor. The developed sensing system revealed for the first time what exactly happens in the extracellular space shortly after calcium oxalate crystals land on renal cells.

2. Materials and methods

2.1. Reagents

3,3′-Dithiodipropionic acid (≥99.0% purity), cytochrome *c* (from bovine heart, 95% purity), xanthine oxidase (XOD, from bovine milk, grade III, 16 mg protein ml^{-1}, 1 U mg^{-1} protein), hypoxanthine (≥99.0% purity), superoxide dismutase (SOD, bovine, 4140 U mg^{-1} protein), phorbol 12-myristate 13-acetate (PMA, approx. 95% purity), glutaraldehyde (25%), HEPES (≥99.5% purity), potassium chloride (≥99.0% purity), calcium chloride (93% purity), and sodium chloride (≥99.5% purity) were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC, 98% purity), and *N*-hydroxysuccinimide (NHS, ≥97% purity) were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Sulfuric acid (95–97%) and hydrogen peroxide (35%) were purchased from Merck KGaA (Darmstadt, Germany). Potassium hydroxide (≥85% purity) was purchased from Lach-Ner s.r.o. (Neratovice, Czech Republic). Calcium oxalate (99.999% purity) was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, USA). All aqueous solutions were prepared using ultrapure water from a Direct-Q® 3 UV system from Millipore S.A.S. (Molsheim, France).

2.2. Cells

Experiments were carried out on monolayers of A6 cells (a kind gift from Dr. W. Van Driessche, Catholic University of Leuven, Belgium) grown on glass coverslips (20 mm × 20 mm × 0.17 mm, Carl Roth GmbH, Karlsruhe, Germany) at 28 °C, and 1% CO_2 in a humidified incubator. Growth medium was renewed twice weekly and consisted of a 1:1 mixture of Leibovitz's L-15 (Gibco brand, catalog no. 31415, from Invitrogen, Carlsbad, CA, USA) and Ham's F-12 media (Gibco, catalog no. 31330, Invitrogen), supplemented with 10% fetal bovine serum (Gibco, catalog no. 2012-11, Invitrogen), 2.6 mM $NaHCO_3$ (Gibco, catalog no. 25080, Invitrogen), 3.8 mM glutamine (Gibco, catalog no. 25030, Invitrogen), 95 U ml^{-1} penicillin and 95 μg ml^{-1} streptomycin (Pen Strep, Gibco, catalog no. 15070, Invitrogen). For the electrochemical experiments, the coverslips were removed from the growth medium, and immersed into prewarmed Ringer buffer (pH 7.4, consisting of 100 mM NaCl, 5 mM HEPES, 1 mM $CaCl_2$, and 2 mM KCl).

2.3. Superoxide biosensor and its operation

Superoxide biosensors were fabricated by a procedure that is detailed in the Supplementary Material, and that is a combination of several previously described methods (such as Manning et al., 1998, Ge and Lisdat, 2002 and Chen et al., 2008b).

Before measuring at cellular level, every superoxide sensor was calibrated. Mixtures of a constant concentration of hypoxanthine (100 μM) and increasing concentrations of XOD (5–50 mU ml^{-1}) were used to generate different concentrations of superoxide and calibrate the superoxide sensors. Both the calibration experiments and the measurements of superoxide release from cells were carried out using a VSP potentiostat equipped with a low-current module (Bio-Logic S.A.S., Claix, France).

2.4. Measuring chamber and its operation

To obtain a convenient measuring chamber, the lid of a 6-well cell culture plate was modified to accommodate the whole electrode system (including the superoxide biosensor, see details in the Supplementary Material). In a typical experiment, a coverslip with cells is immersed in the well containing Ringer buffer, the culture plate is then closed with the lid bearing the electrode system, and the superoxide sensor is lowered in the solution, before being polarized at +150 mV. After the current of the sensor decreases to a stable background value, a certain amount of calcium oxalate crystals is injected into the well, and uniformly dispersed allover the cell layer by stirring (for about 20 s). If the cells release superoxide, the current signal of the sensor increases proportionally with the superoxide concentration. New sensors were used for each cellular experiment.

The appropriate temperature during experiments was assured by blowing warm air from a fan heater into a polystyrene box enclosing the above measuring chamber. The home-made environmental chamber was computer controlled.

2.5. AFM experiments

AFM imaging was conducted with a NanoWizard II instrument (JPK Instruments, Berlin, Germany). Cells were fixed using a 2.5% glutaraldehyde solution for 15 min before imaging. Imaging of cells was performed in Ringer buffer. Topographic images were taken in contact mode using the cantilever with a spring constant of 0.3 N m^{-1} (MikroMash, Tallinn, Estonia). The force applied to the cantilever was just sufficient to remain in contact with the surface (and corresponding to a setpoint of maximum 0.3–0.5 V). The scan rate was 0.3 Hz.

3. Results and discussion

3.1. Characterization of the superoxide sensor

The cyclic voltammograms of the constructed superoxide sensors displayed a pair of current peaks around a formal potential of −51 ± 5 mV (mean ± standard deviation for 11 different sensors; data not shown). This formal potential and the linear increase of the peak height with the scan rate allowed us attribute the current peaks to the oxidation and reduction of surface-immobilized cytochrome *c*. The surface concentration of the immobilized cytochrome *c* (11.7 ± 2.9 pmol cm^{-2}; mean ± standard deviation for 12 different sensors), calculated from the charge under the cathodic peak, corresponds to a monolayer of immobilized cytochrome *c* (Ge and Lisdat, 2002; Tammeveski et al., 1998).

Before attempting to measure the release of superoxide by cells, how the cytochrome *c*-modified electrodes behave in the presence of enzymatically generated superoxide was investigated. The following four properties are worth mentioning in view of subsequent experiments on cells:

(1) After establishing a certain concentration of superoxide, the sensors reach a steady-sate current signal in 27.1 ± 11.4 s (mean ± standard deviation for 30 signals on 10 different sensors). The developed system allows thus the observation of oxidative burst from cells with a time resolution better than 1 min.
(2) The sensitivity of our biosensor was determined to be $18.3 \pm 2.7 \times 10^2$ A m^{-2} M^{-1} (mean ± standard deviation for 12

investigated sensors; see calibration curve in Figure 2 of Supplementary Material). This sensitivity is higher than the sensitivity of some other SAM-based superoxide biosensors (Ge and Lisdat, 2002; Tammeveski et al., 1998), and smaller than the sensitivity reported in the work that motivated the selection of 3,3′-dithiodipropionic acid as linker between electrode and cytochrome *c* (Chen et al., 2008b). It also allows converting signals observed at cellular level into absolute superoxide concentrations.

(3) The biosensor exhibits good linearity up to 1 μM superoxide. This linear range was never exceeded by superoxide concentrations observed at cellular level.

(4) Current signals observed during calibration were significantly diminished (85–100%) when adding SOD (i.e. an enzyme consuming superoxide) into the measuring chamber. The current signal given by 50 mU ml^{-1} XOD and 48 μM hypoxanthine decreases back to its background value after adding 62 U ml^{-1} SOD to the reaction mixture (Figure 3 of Supplementary Material). This proves that most of the measured current is indeed due to reduction of cytochrome *c* by superoxide.

In the next step, superoxide sensors were used to observe whether renal cells challenged with calcium oxalate crystals release superoxide into their extracellular space or not.

3.2. Observing superoxide release from A6 cells

The electrode system for the detection of superoxide was assembled into the lid of a 6-well cell culture plate to assure compatibility with plasticware commonly used in cell culture laboratories (Figure 1 of Supplementary Material). This design minimized the time between the removal of cells from incubator and their investigation with the superoxide biosensor. Moreover, it allowed an easy and reproducible positioning of the sensor in the close proximity of the cells.

A6 cells were selected for the present study because of two reasons. They are not only derived from kidney (distal tubules of *Xenopus laevis*) but they also do not require working at 37 °C (as mammalian cells do), and thus they are very convenient to work with.

There are three important features to note about the current recorded after dispersing calcium oxalate crystals over A6 cells (Fig. 1). First, within minutes (5–6 min) after adding calcium oxalate, the cells started to secrete superoxide that was sensed by the sensor. Second, in about 20 min the superoxide-proportional signal passes through its maximum value. The third important feature of the current is that after about 3 h it returns to the background value indicating that the superoxide release stopped, and the extracellular superoxide was cleared away. In conclusion, the release of superoxide by renal cells challenged with calcium oxalate crystals is a temporary, nonmonotonous process. Therefore, the usefulness of assays which measure the concentration of short-lived ROS at the end of a few hours long incubation is questionable. How much a cell suffered of oxidative stress can correctly be evaluated only by a continuous monitoring.

The next step was to confirm that the recorded current is indeed due to superoxide. When adding SOD up to 318 U ml^{-1} the signal rapidly decreased from 2.6 nA to 0.3 nA suggesting that more than 90% of the signal is given by superoxide (Fig. 1). For comparison an experiment performed in similar conditions but without additions of SOD is also shown in the figure. In such experiments the current signal decreased much slower and exclusively due to the uncatalyzed dismutation of superoxide.

A6 cells were also exposed to PMA following an exposure to calcium oxalate crystals in order to identify the possible source of superoxide. PMA is a compound known to activate membrane

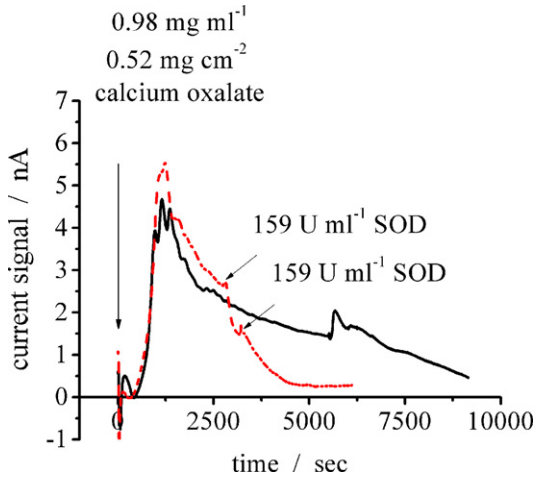

Fig. 1. Superoxide release by A6 renal cells exposed to calcium oxalate crystals (black solid line) or calcium oxalate crystals and then SOD (red dashed line). *Conditions*: Ringer buffer as supporting electrolyte; +150 mV applied potential; room temperature; the moment of injecting the crystals was selected as zero of the time scale; the current signal was background corrected and smoothed using the adjacent averaging method with a 10 points moving window. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

bound NAD(P)H oxidase through the protein kinase c pathway (and thus to cause superoxide production) (Lundqvist et al., 1996). PMA caused the release of additional superoxide in the extracellular space (Fig. 2). This release was longer than any superoxide release induced by calcium oxalate. Since the effect of calcium oxalate crystals is mimicked by PMA, NAD(P)H oxidase might be also the source of the superoxide observed at A6 cells exposed to calcium oxalate.

3.3. Effect of cell age on the superoxide release

A6 cells grow to confluence in few days after they are seeded. After about 7 days, they also form so called tight junctions (Jaeger et al., 1997). Tight junctions are claudin- and occluding-based leak

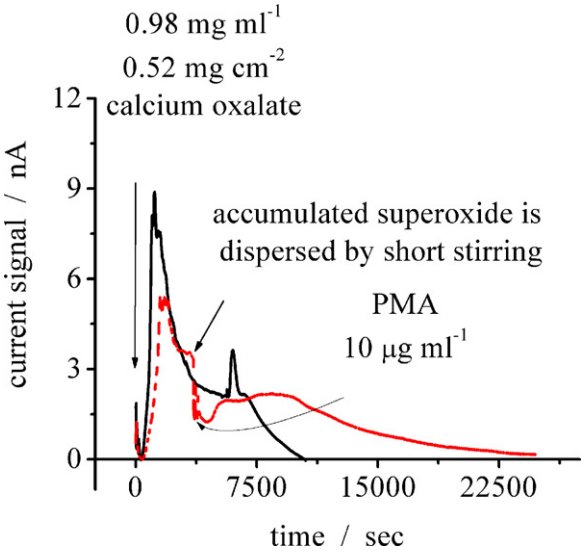

Fig. 2. Release of additional superoxide by A6 renal cells exposed also to PMA after being challenged with calcium oxalate crystals. Current signals for cells exposed only to calcium oxalate crystals (black solid line) or to calcium oxalate crystals and then PMA (red dashed line) are shown. *Conditions*: temperature of 28 °C; other conditions as in Fig. 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

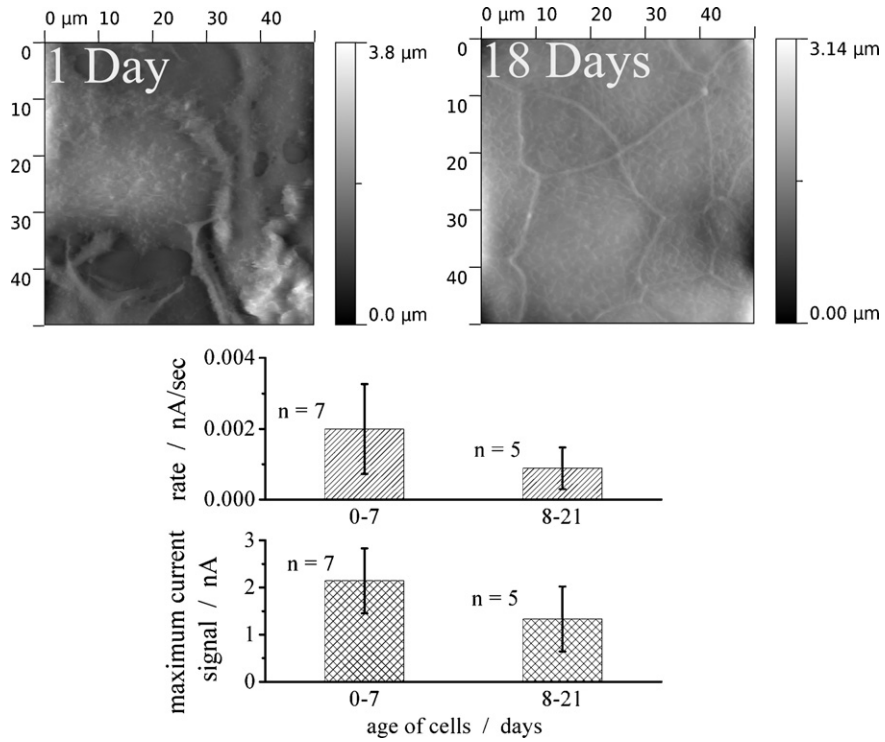

Fig. 3. Time evolution of the cell morphology as revealed with AFM (upper row), and of the maximum current and rate of current increase observed at calcium oxalate-challenged A6 cells (lower row). *Conditions*: temperature of 28 °C; A6 renal cells were challenged with 0.98 mg ml^{-1} (0.52 mg cm^{-2}) calcium oxalate; other conditions as in Fig. 1.

proof seals between adjacent cells (Balkovetz, 2009; Giepmans and van Ijzendoorn, 2009). These special accumulations of proteins are expected to give not only mechanical robustness to the cell layer but also an increased ability to withstand chemical challenges such as the one by calcium oxalate crystals. To check this hypothesis, cells of two different ages (<7 days, and >7 days) were challenged with the same amount of calcium oxalate. The time evolution of both the cell morphology (as revealed with AFM) and the current given due to superoxide release is shown in Fig. 3. The AFM images revealed that cells are fully covering the glass coverslip already next day after seeding but without having tight junctions (upper left corner of Fig. 3), and also that cells older than 7 days are indeed strongly united by tight junctions (upper right corner of Fig. 3). In the same time, the maximum current and the rate of the current increase are both indicating that A6 cells in their first week release larger amounts of superoxide faster than A6 cells in their second and third week of culturing. It seems that in the absence of tight junctions the calcium oxalate has access to a larger part of the cell surface. Tight junctions, once formed, protect the basolateral side of renal cells (leaving exposed to calcium oxalate only the apical side), and this causes the superoxide release to decrease. Using other renal cells and a chemiluminescence-based method, the calcium oxalate mediated intracellular superoxide production has already been observed to decrease about 40% when cells had been confluent for more than 7 days (Khand et al., 2002).

3.4. Effect of calcium oxalate concentration on the superoxide release

Once the effect of cell maturity was clarified, this experimental parameter was kept constant and the amount of calcium oxalate crystals was changed. Results show that A6 cells do release more superoxide when challenged with more calcium oxalate crystals (Fig. 4). The recorded dose–effect curve indicates a maximum effect at 0.5 mg ml^{-1} (0.26 mg cm^{-2}) calcium oxalate. The amount of released superoxide decreases again at higher oxalate concentrations. Cellular viability might be affected at such high concentrations. Data from literature indicate that only the smallest concentration of calcium oxalate used in the present study did not affect cell viability of MDCK cells (derived from canine kidney tissue) or NRK-52E cells (a rat renal epithelial cell line) (Escobar et al., 2007; Semangoen et al., 2008). Calcium oxalate in concentrations comparable with 1 mg mL^{-1} increased the percentage of dead cells to 27.5 [in human proximal tubule cells (Guo and McMartin, 2005)], 28 [in MDCK (Thongboonkerd et al., 2008)], 81.25 [in proximal tubule cells from Wistar rats (Guo and McMartin, 2005)] or 90 [in proximal tubule cells from F-344 rats (Guo and McMartin, 2005)]. However, such high percentages of cell death are the result

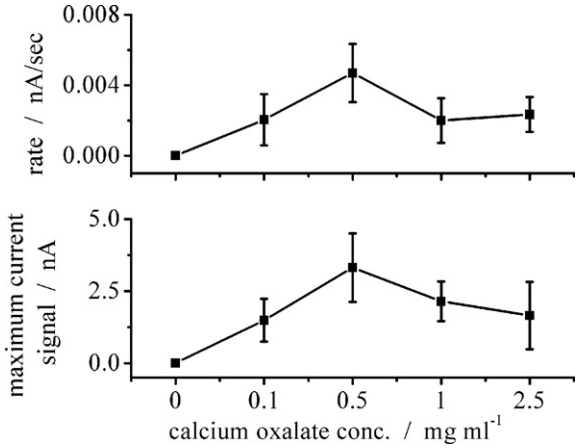

Fig. 4. Effect of the amount of crystals on the superoxide release from A6 cells challenged with calcium oxalate. The maximum current and the rate of current increase observed in 20 experiments versus the final concentration of the calcium oxalate in the measuring chamber are shown. *Conditions*: temperature of 28 °C; A6 renal cells at the age of 2–7 days were used; other conditions as in Fig. 1.

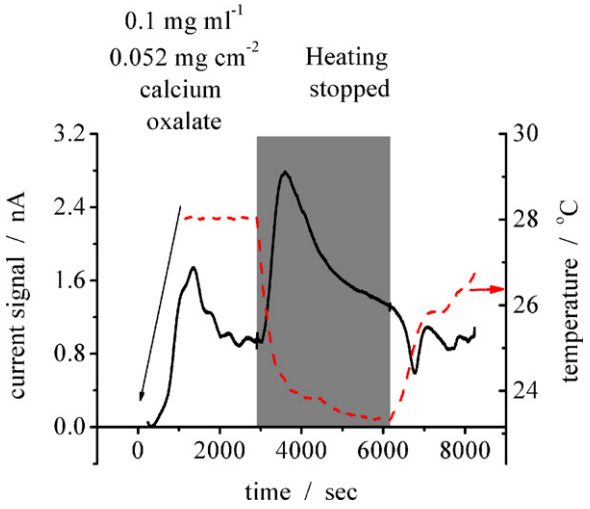

Fig. 5. Superoxide release by A6 renal cells exposed to calcium oxalate crystals and then a temperature drop of about 5 °C. *Conditions*: as in Fig. 1, excepting temperature, which was modulated as shown.

of much longer incubation times than used in our experiments (see Table 1 of Supplementary Material).

3.5. Effect of temperature on the superoxide release

A6 cells are normally cultured at 28 °C. Therefore, we supposed that moderate changes in temperature are not important, and initial experiments were carried out at room temperature (23–25 °C). In a similar manner, for experimental convenience, the majority of amperometric studies on exocytotic processes are performed at room temperature and not under physiological conditions (Amatore et al., 2008). In order to reveal whether moderate temperature changes are important or not, the temperature was more carefully controlled. In a typical experiment, after challenging the cells with calcium oxalate crystals at 28 °C, the temperature was dropped to room temperature. This decrease of about 5 °C surprisingly appeared as a significant stress for the cells indicated by the appearance of a second oxidative burst (Fig. 5). Judging from the amplitude of the current signal, a temperature drop of about 5 °C causes a more significant cellular discomfort than the addition of 0.1 mg ml^{-1} (0.052 mg cm^{-2}) calcium oxalate crystals. The observed current change is also much higher than the one observed without cells (i.e. exclusively on the sensor, see Figure 4 of Supplementary Material). It is thus clearly due to the cells. Therefore, it is quite important to carefully control the temperature when investigating oxidative stress at cellular level in order not to induce additional stress. Temperature changes before the actual experiment (i.e. while removing cells from the incubator, during washing, etc.) should also be carefully avoided for reproducible observations.

4. Conclusions

The appearance of superoxide in the extracellular space of renal cells challenged with calcium oxalate crystals was for the first time continuously monitored using an amperometric biosensor. It was observed that superoxide appears in the extracellular space within minutes after A6 renal cells encounter calcium oxalate crystals. The amount of released superoxide depends on the presence of superoxide-scavenging enzymes, the age of the cells, the amount of crystals, and the temperature. Superoxide release induced by calcium oxalate crystals in renal cells is a nonmonotonous, temporary process. Therefore, unlike the developed sensing system, assays measuring short-lived ROS at cellular level at the very end of long incubation times cannot appropriately reveal the extent of oxidative stress suffered by the cells. The obtained results prove that the developed sensing system can be a useful tool for investigating the biochemistry behind kidney stones. In combination with inhibitors of the superoxide production pathways [e.g. rotenone or piericidin which are specific complex I inhibitors (Lambert and Brand, 2004), or apocynin that is an NADPH oxidase inhibitor (Dodd-O and Pearse, 2000)], it can be used to reveal the exact subcellular source of the superoxide produced by renal cells incubated with calcium oxalate crystals. Studies using different renal cell lines and more traditional cellular biology approaches have already indicated both NAD(P)H oxidase (Thamilselvan et al., 2009) and mitochondria (Khand et al., 2002; Meimaridou et al., 2005) as the source of the superoxide induced by calcium oxalate. Arginine methyl ester (Ozturk et al., 2006) and epigallocatechin 3 gallate (Itoh et al., 2005) were discovered to decrease free radical-mediated injury in experimental nephrocalcinosis induced in animal models and in hypoxia induced in cellular models, respectively. The superoxide sensing system could also be useful in similar attempts to identify and study substances which can reduce the cell injury caused by calcium oxalate crystals. Combined or not with other cellular models, it could also be used for testing the toxicity of other particulate material (such as engineered nanoparticles). The negative impact nanoparticles have on living cells is also very often mediated by oxidative stress that is due to overproduction of ROS (such as superoxide) and that is causing DNA and protein damage (Eom and Choi, 2009; Foldbjerg et al., 2009; Hussain et al., 2005; Kim et al., 2009). Nanoparticles may generate ROS directly (Foucaud et al., 2007) or indirectly (by altering the function of mitochondria or NADPH oxidase) (Ad et al., 2004).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.12.013.

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