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Synoviocytes, not chondrocytes, release free radicals after cycles of anoxia/re-oxygenation

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Abstract

By oxymetry and electron paramagnetic resonance (EPR), we investigated the effects of repeated anoxia/re-oxygenation (A/R) periods on the respiration and production of free radicals by synoviocytes (rabbit HIG-82 cell line and primary equine synoviocytes) and equine articular chondrocytes. Three periods of 20 min anoxia followed by re-oxygenation were applied to 10^7 cells; O_2 consumption was measured before anoxia and after each re-oxygenation. After the last A/R, cellular free radical formation was investigated by EPR spectroscopy with spin trapping technique ($n = 3$ for each cell line). Both types of synoviocytes showed a high O_2 consumption, which was slowered after anoxia. By EPR with the spin trap POBN, we proved a free radical formation. Results were similar for equine and rabbit synoviocytes. For chondrocytes, we observed a low O_2 consumption, unchanged by anoxia, and no free radical production. These observations suggest an oxidant activity of synoviocytes, potentially important for the onset of osteoarthritis.

Keywords: Equine; Articular chondrocytes; Synoviocytes; Free radical; Electron spin resonance; Anoxia

Synovitis is commonly considered to be an initial change in joints of athletic horses and associated to repeated trauma. Ischemic conditions have been evoked as secondary consequence of joint effusion in the follow-up [1]. In 1989, it was pointed out that movements of an inflamed joint could lead to ischemia/reperfusion injury and might lead to the persistence of inflammatory joint disease by the local production of oxygen free radicals [2]. If acute or chronic trauma could already alter the blood supply of a sound synovial membrane, leading to cycles of ischemia/reperfusion (via edema and transient tissue hypoxia), this might start the production of reactive oxygen species (ROS) and could participate by intracellular and extracellular oxidative damage to progressive cartilage degradation and osteoarthritis. In a traumatic joint submitted to anoxia/re-oxygenation (A/R), both synoviocytes and chondrocytes could be theoretically responsible for ROS production. Articular chondrocytes in vivo live under poor oxygen (O_2) tension [3]. We already observed that equine articular chondrocytes in culture could survive for more than 10 days to near anoxia conditions (<1% O_2 in the surrounding atmosphere) [4], and we recently observed that the O_2 uptake of equine articular chondrocytes was very low (20.5 pmol O_2/min/10^6 cells), even if they were cultured

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at 21% O_2 during 48 h before the assays. Their respiratory rate did not increase after an anoxia period of 30 min (data submitted for publication).

The synoviocytes are the second kind of articular cells which we can suppose as implicated in ROS production, at least under stimulating conditions such as A/R or acute inflammation. But, no data are available in literature about the ROS producing capacity of synoviocytes.

So we were interested to study the capacity of equine articular chondrocytes and synoviocytes to produce free radicals in A/R conditions. To reach this goal, we used equine articular chondrocytes and synoviocytes (rabbit synoviocyte cell line HIG-82 and synoviocytes isolated from horse joint) and oxymetry coupled to electron spin resonance spectroscopy (EPR) in combination with the spin trapping technique. We previously designed an A/R model for chondrocytes (data submitted for publication, see above). A similar model was established for synoviocytes. With these chondrocyte and synoviocyte models, we tried to demonstrate whether synoviocytes or chondrocytes had different respiration rates and were able to produce free radicals when submitted to A/R cycles.

Materials and methods

Reagents. PenStrep (penicillin 10^4 IU/ml, steptomycin 10 mg/ml) and Hepes were from Cambrex (Verviers, Belgium). Collagenase (type IA, *Clostridium histolyticum*) and bovine testicular hyaluronidase type IV-S were from Sigma–Aldrich (Bornem, Belgium). Amphotericin B (250 μg/ml), gentamycin (50 mg/ml), proline, glutamine, DMEM (Dulbecco's modified Eagle's medium) with 1 g/L glucose (without phenol red), Ham's F 12, trypsin/EDTA in HBSS, and foetal bovine serum (FBS) were purchased from Invitrogen (Merelbeke, Belgium). A working solution of special mix of antibiotics (antibiotic special mix: 2.5 μg/ml polymixin B, 5 μg/ml gentamycin, and 5 μg/ml vancomycin) was prepared either in DMEM or Ham's F 12 milieu. Ethanol (EtOH) and the spin trap agent, α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone (POBN), were purchased from Aldrich (Belgium). Pronase (type E of *Streptomyces griseus*) and analytical grade sodium, potassium, calcium, and magnesium chlorides, magnesium sulphate, sodium hydrogen carbonate and hydrogen phosphate, potassium dihydrogen phosphate, and glucose were from Merck (VWR International, Leuven, Belgium). Trypan blue was from ICN Biomaterials (Eschwege, Germany). Gases were obtained from Air Liquide (Belgium). Spin trap was dissolved in Hanks' balanced salt solution added with glucose (HBSS-G: 138 mM NaCl, 5.4 mM KCl, 4 mM $NaHCO_3$, 34 mM Na_2HPO_4, 0.33 mM KH_2PO_4, 0.4 mM $MgSO_4{\cdot}7H_2O$, 0.5 mM $MgCl_2{\cdot}6H_2O$, 1.4 mM $CaCl_2{\cdot}H_2O$, and 5.6 mM glucose; pH 7.4).

Chondrocyte and synoviocyte isolation from equine joint. The articular chondrocytes and synoviocytes were harvested from the stifle joints of two trotting horses (4 and 5 years) euthanised for welfare reasons, but without orthopedic problems. An area of about 20×20 cm at the stifle was clipped and the skin was removed without preliminary opening of the joint cavity. The joint capsule was aseptically opened and the synovial membrane was carefully dissected. The recovered pieces were cut to small pieces (4 mm^2) and submitted to an enzymatic digestion (3.5 h) in around 20 ml of the antibiotic special mix in Ham's F12 nutrient mixture enriched with 1% PenStrep, 1% amphotericin B, and 0.75 mg/ml of type IA of *Clostridium histolyticum*.

The articular chondrocytes were isolated aseptically as previously described [4]. Briefly, the cartilage was cut in small pieces and submitted to a triple enzymatic digestion (sequentially: 1100 IU/ml hyaluronidase, 0.5 mg/ml pronase, and 1 mg/ml collagenase) in around 20 ml of the antibiotic special mix in DMEM, added with 10 mM Hepes, 10% heat-inactivated FBS, 2 mM proline, 200 μg/ml glutamine, and 0.5 μg/ml amphotericin B.

After digestion, the synoviocyte and chondrocyte suspensions were filtered (cell strainer porosity 70 μm), centrifuged (5 min at 1000 rpm), and washed three times in their respective culture medium. The cells were counted and their viability was quantified by trypan blue exclusion (0.4% in buffered saline solution) before further culture.

Culture conditions. The rabbit synoviocyte cell line (HIG-82; LGC Promochem SARL Molsheim France) and the equine synoviocytes were cultured in Ham's F12 medium supplemented with 10% heat-inactivated FBS, 0.2% gentamycin, 1% PenStrep, and 1% amphotericin B at 37 °C under humidified atmosphere (5% CO_2 and 95% air). Prior to the assays, the monolayered synoviocytes were detached by incubation at 37 °C with a trypsin/EDTA solution (4 min incubation for rabbit cells and 6 min for equine primary synoviocytes). The cells were distributed at 10^7 per tube, centrifuged at 400*g* for 5 min, and the cell pellets were immediately used for oxymetry and EPR assays (see below).

The articular equine chondrocytes were distributed at 10^7 cells per 20 ml in Falcon tubes and cultured in DMEM containing 10% heat-inactivated FBS, 2 mM proline, 200 μg/ml glutamine, 1% PenStrep, 10 mM Hepes, and 1% amphotericin B. The medium has been adjusted to 5% O_2, 5% CO_2, and 90% N_2 by bubbling during 30 min with a prepared gas mixture. The Falcon tubes were enclosed in an airtight container with an O_2 gas phase tension of 5%, in the presence of 5% CO_2, and 90% N_2 in a humidity-saturated atmosphere 2 days before the oxymetry onset. The samples were cultured at 37 °C on an orbital shaker (70 rpm). Cell handling for the culture at 5% O_2 was performed in a sterile glove box (glove box C45201; Fisher Bioblock, Tournai, Belgium), gas-controlled (N_2, O_2, and CO_2) by micro flow rotameters. The O_2 content of the glove box was monitored with an oxymeter (Oxycom 100 D, Germany). After this preconditioning culture period, the tubes were centrifuged at 400*g* for 5 min. The cell number was readjusted to 10^7 per tube (with a viability determined by trypan blue exclusion superior to 95%) and the cell pellets were recovered for the oxymetry and EPR assays.

Oxymetry and anoxia/re-oxygenation. The O_2 consumption by 10^7 cells (synoviocytes or chondrocytes) was monitored in 2 ml of air-saturated HBSS–glucose, in the presence of 50 mM POBN and 2% EtOH (v/v), with a Clark electrode, at 37 °C. The kinetics of O_2 consumption was recorded with an oxygraph (Oroboros oxygraph, Paar Physica, Austria). The measurement started just after transferring the mixture in the respiration chamber (test chamber) and closing it. As control assays, 10^7 cells were put in the same mixture in the second chamber of the oxymeter (control chamber), and their normal respiration rate was monitored without closing the chamber. The A/R assays were performed as previously described [5]. The synoviocytes (cell line and primary equine cells) reached anoxia after complete consumption of the O_2 in the medium, while for chondrocytes, the anoxia sequence was reached by bubbling the medium with N_2. Anoxia was maintained during 20 min, and followed by opening of the test chamber and re-oxygenation performed by exposure of the stirred medium to air until O_2 saturation. Afterwards, the respiration chamber was again closed, the respiration rate was monitored and a new cycle of A/R was performed. Three cycles of A/R were performed. For equine articular chondrocytes, each period of anoxia was reached by a new bubbling with nitrogen. At the end of the third anoxia period, the samples were subjected to EPR measurements (see below), and the cellular viability was determined by trypan blue exclusion. Oxymetry assays were done in triplicate for each cell line. The slopes of O_2 consumption were calculated with the Orobos oxygraph included software.

EPR spin trapping experiments. After the third sequence of anoxia, the cell suspension was transferred into the quartz flat cell and put into the cavity of the EPR spectrometer. The presence of free radicals was evaluated by following the production of POBN/ethoxy radical adducts. All measurements were carried out at room temperature with a Bruker spectrometer (Bruker, Karlsruhe, Germany), operating at X-band frequency (9.75 GHz) and at a microwave power of 20 mW. The instrumental settings were the following: 100 kHz modulation frequency, 1.012 G modulation amplitude, 3480 G magnetic field centre, and receiver gain was 2×10^4. The sweep width was 100 G and the total number of scans was 6 or more. The hyperfine splitting constants were measured from the experimental spectra by means of a Bruker Win-Simfonia program running under Microsoft Windows. The assays were repeated three times for each cell line.

Results

Cell isolation and culture

By enzymatic digestion from cartilage and synovial membranes, we isolated chondrocytes and synoviocytes with a viability $\geqslant 95\%$. The chondrocytes were cultured for 48 h before the oxymetry and A/R assay: during this period, they maintained their phenotype, and their number and viability remained unchanged. Before oxymetry, the synoviocytes were cultured until confluence; after trypsination, their viability was $\geqslant 95\%$.

Respiration rate of joint cells and effects of anoxia

Both, the rabbit and the primary equine synoviocytes presented the same respiratory rate, as shown in Fig. 1. We observed that 10^7 synoviocytes completely consumed O_2 within 20 min, with a respiration slope around 11 μmol O_2/min as presented in the typical example of Fig. 1 (curve 1). The A/R cycles slowered the respiration rate (Fig. 1, curves 2–4): the first anoxia period decreased the respiration slope to 5.55 μmol O_2/min (50% decrease), and the decrease continued after the second and third anoxia periods but less accentuated, with respiration slopes of 4.0 and 3.22 μmol O_2/min, respectively. After the last period of anoxia, the shape of the curve changed (loss of linearity) indicating mitochondrial damage. Viability of the cells at the end of the experiment was $\geqslant 75\%$. The respiration rate of 10^7 equine articular chondrocytes was linear but the consumption of O_2 was very weak (0.14 μmol O_2/min), so that the chondrocytes never reached anoxia spontaneously (Fig. 2): a bubbling with N_2 was needed. The effects of repeated cycles of A/R on their respiration rate were negligible without clear sign of slope change and thus no mitochondrial damage. The chondrocyte viability after the experiment was superior to 90%.

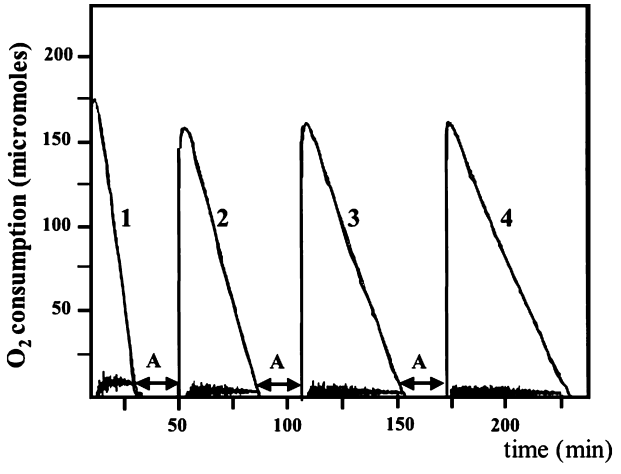

Fig. 1. Oxymetry of 10^7 synoviocytes (HIG cell line) and effects of anoxia/re-oxygenation cycles. A indicates anoxia and the two-headed arrow the period of anoxia. Four slopes (1–4) of O_2 consumption were calculated: 11.0, 5.5, 4.0, and 3.2 μmol O_2/min respectively.

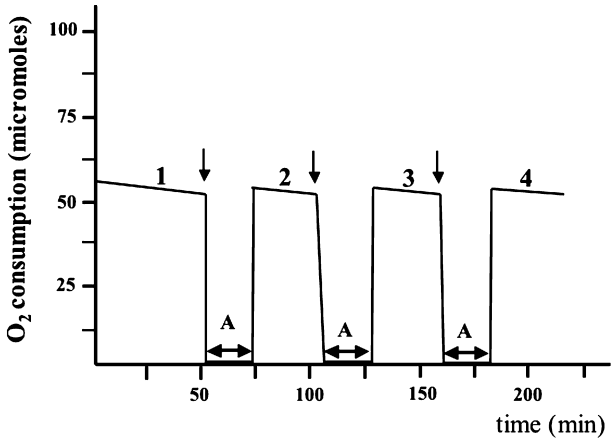

Fig. 2. Oxymetry of 10^7 equine chondrocytes and effects of anoxia/re-oxygenation cycles. A indicates anoxia and the two-headed arrow the period of anoxia. Arrow: N_2 bubbling (necessary to reach anoxia).

Free radical release observed by EPR: spin trapping studies

EPR studies were performed with POBN with and without addition of EtOH. Normoxic control cells (10^7 equine articular chondrocytes, HIG-82 rabbit synoviocytes or primary equine synoviocytes) in 2 ml HBSS-G, supplemented with POBN or POBN/EtOH mixture did not show free radical signals. After three cycles of A/R, no free radical formation was observed in equine articular chondrocytes, but on the contrary, the EPR spectra of both types of synoviocytes, rabbit HIG-82 and primary equine synoviocytes, evidenced the production of free radicals (Figs. 3 and 4) and showed six-line EPR spectra characteristic of POBN/$^{\bullet}$CH(OH)CH_3 adducts with the following coupling constants: $a_N = 15.7$ G and $a_H = 2.7$ G (Figs. 3B and 4B). The EPR signal was more intense for primary equine synoviocytes (Fig. 4B). In contrast, after A/R cycles without the addition of ethanol, synoviocytes showed only

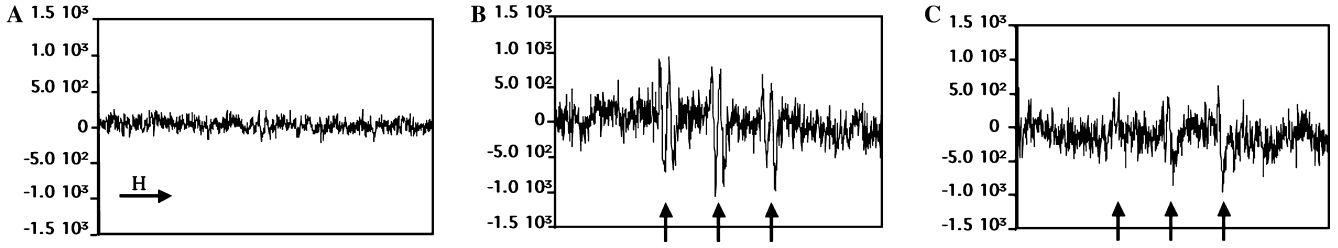

Fig. 3. EPR signal of 10^7 rabbit synoviocytes obtained at the end of three anoxia/re-oxygenation cycles. Arrows indicate the signal lines. (A) Control (cells in normoxia), (B) POBN/ethoxy adduct, and (C) lipid radical production (POBN/L adduct).

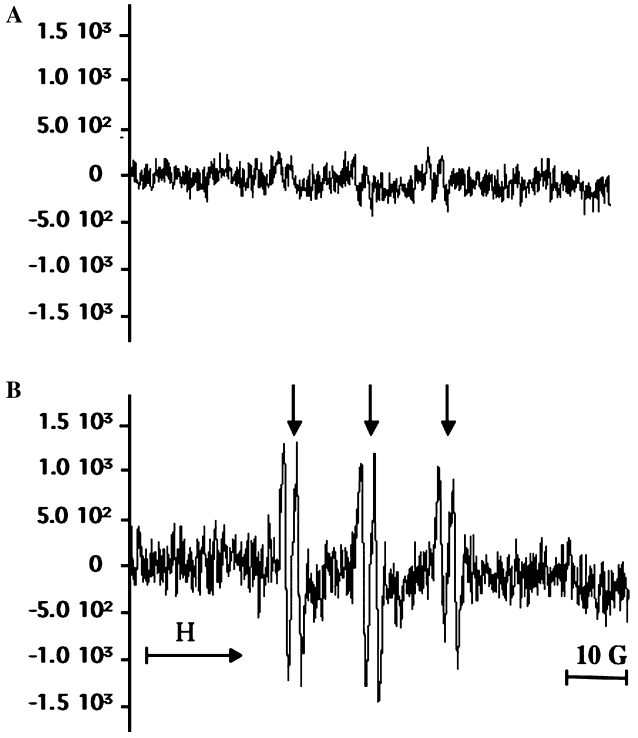

Fig. 4. EPR signal of 10^7 primary equine synoviocytes obtained at the end of three anoxia/re-oxygenation cycles. Arrows indicate the signal lines. (A) Control (cells in normoxia) and (B) POBN/ethoxy adduct.

three-line EPR spectra (POBN/lipid adduct, $a_N = 15.2$ G), suggesting carbon-centred radicals derived from lipid peroxidation (Fig. 3C).

Discussion

The oxymetric assays of synoviocytes in suspension indicated that the respiratory function of the cells was altered by repetitive cycles of A/R, and by EPR spin trapping methodology we found that free radicals were produced. Indeed after each cycle of A/R, the synoviocytes needed more time to consume the O_2 of the medium before they achieved again anoxia. This effect can partially be attributed to a reduced viability, but after the last anoxia period, we also observed a parabolic shape in the oxymetric curves of the synoviocytes, indicating a disturbed mitochondrial chain activity. In our study, exogenous compounds known to stimulate cells were not added. The production of free radicals by synoviocytes was the result of a stimulation by cycles of A/R, an observation that has never been reported previously. The synoviocytes build or live at the border between joint cavity and joint capsule. They are exposed to >12% O_2 saturation of the arterial blood whereas the chondrocytes live under hypoxic conditions (about 7% O_2 in the synovial liquid) [3]. These life conditions suggest that synoviocytes are more susceptible to A/R than articular chondrocytes. Our oxymetry and EPR studies confirm this hypothesis, demonstrating a sensibility towards a lack of O_2 and an oxidant activity of synoviocytes at least related to ROS production. EPR spectra of POBN/ethoxy adducts were observed during A/R of isolated liver mitochondria and were attributed to the reaction of hydroxyl radicals, originating from superoxide anion, with the POBN/EtOH couple [6]. POBN can cross the mitochondrial membranes and reach the matrix compartment. The EPR signals that we reported here were similar to those obtained with isolated mitochondria, and suggested a mitochondrial origin: the observed EPR spectra of POBN/ethoxy adducts can be attributed to the reaction of superoxide anion by-product with ethanol in the presence of the spin trap agent [7]. Indeed, mitochondria are reported to produce superoxide anion and other ROS in normal as well as in pathological conditions of A/R [7–9]. The superoxide anion that is produced on the matrix side of the mitochondrial membrane can cause cell damage (e.g., lipid peroxidation) by reacting with metal iron complexes within the enzymatic domain. Superoxide anion was also described as able to attack the aconitase, releasing the labile iron of the cluster $[4Fe–4S]^{2+}$ and forming Fe^{2+} [10]. Superoxide anion also produces hydrogen peroxide (H_2O_2) by subsequent dismutation, and both species, in the presence of Fe^{2+}, may form the highly reactive species hydroxyl radicals which are known to cause cell damage especially by lipid peroxidation.

Superoxide anion can also react with nitric oxide ($NO^{\cdot}$), of which the production is recognised in mitochondria and increases with increased Ca^{2+} concentrations, such as during anoxia [9,11]. From this reaction,

peroxynitrite can be formed, from which new radical species are generated, such as hydroxyl radicals, favouring the peroxidation of membrane phospholipids and generating lipidic radical species.

With 4-POBN, we found spin adducts indicating the presence of other radical species with a longer life period. These radical species could originate from mitochondrial lipid peroxidation, maybe by in situ production of hydroxyl radical or other free radical species. Of course, we cannot exclude a cytosol origin of superoxide anion and derived species, by the anoxia-activated xanthine oxidase, but the simultaneous demonstration of an altered respiratory function suggested that the mitochondrial respiratory chain was damaged and so mainly responsible for the production of free radicals that could be primarily superoxide anions.

However, our study does not permit to say at which moment accurately the formation of free radical takes place. Further investigations are necessary to identify the sequence of radical production and to identify the nature of these radical species. At least, we can suspect the onset of free radical production during anoxia, increasing at re-oxygenation as it has been demonstrated for other cell types [5]. For chondrocytes, after A/R we did not observe change in the respiration rate or free radical production. Their respiration rate was very low, maybe explaining the absence of free radical at the mitochondrial level.

Tiku et al. [12] showed the production of superoxide and hydrogen peroxide by chondrocytes, but after stimulation by phorbol 12-myristate 13-acetate in the presence of ferrous iron. They also found a hydroxyl formation by unstimulated rabbit and human cartilage in the presence of ferrous iron. Their working conditions were comparable to inflammation conditions, which are considered as accompanied by reactive oxygen species production [13]. Our working conditions were different since we used A/R without another stimulator. However, as we observed that synoviocytes formed ROS under A/R conditions, we cannot exclude that, in vivo, the production of reactive oxygen species by synoviocytes could damage iron binding proteins transferrin and ferritin and contribute to stimulate chondrocyte to produce ROS, in the presence of released iron.

Conclusions

The results demonstrated that A/R of primary equine and immortalised rabbit synoviocytes led to respiratory dysfunction and to the production of free radicals. The free radicals seemed to originate, at least partially, from damaged mitochondria, but this hypothesis needs further investigation. On the contrary, primary equine articular chondrocytes did not show altered respiration patterns and formation of free radicals when exposed to anoxia. These observations indicate an important role of synoviocytes in the onset of osteoarthritis and suggest that chondrocytes could be, in a first time, the victims of free radicals built by synoviocytes, before they start their own inflammatory response.

Acknowledgments

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