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Enhanced Mitochondrial Radical Production in Patients with Rheumatoid Arthritis Correlates with Elevated Levels of Tumor Necrosis Factor alpha in Plasma

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Mitochondrial dysfunction contributes to cell damage in a number of human diseases. One significant mechanism by which mitochondria damage cells is by producing reactive oxygen species from the respiratory chain. In this study we measured the production of reactive oxygen species by leukocyte mitochondria in blood from rheumatoid arthritis patients. To do this we used the chemiluminescence of lucigenin, which is accumulated by mitochondria within cells and reacts with superoxide to form a chemiluminescent product. By using specific inhibitors we could distinguish between the production of reactive oxygen species by mitochondria and by NADPH oxidase. There was a five-fold increase in mitochondrial reactive oxygen species production in whole blood and monocytes from patients with rheumatoid arthritis, when compared to healthy subjects or patients with non-rheumatic diseases. There was no increase in mitochondrial reactive oxygen species production by neutrophils from rheumatoid arthritis patients. The enhanced mitochondrial radical production in rheumatoid arthritis patients correlated significantly with increased levels of tumor necrosis factor alpha in plasma ($p<0.0001$). As tumor necrosis factor alpha is known to increase mitochondrial reactive oxygen species production the elevated mitochondrial radical formation seen in rheumatoid arthritis patients may be due to activation of the mitochondrial radical production. These data suggest that elevated mitochondrial oxidative stress contributes to the pathology of rheumatoid arthritis.

Key words: mitochondrial oxidative stress, reactive oxygen species, lucigenin chemiluminescence, rheumatoid arthritis, tumor necrosis factor alpha, respiratory chain inhibitors

Abbreviations: Cl, chemiluminescence; cph, counts per hour; DMSO, dimethylsulfoxide; DPI, diphenylene iodoniumchloride; GSH, gluathione; IL, interleukin; lucigenin, bis-*N*-methylacridinium nitrate; IntD, Internal diseases; Mn-SOD, manganese superoxide dismutase (EC 1.15.1.1); NF-κB, nuclear factor kappa B; RA, rheumatoid arthritis; ROS, reactive oxygen species; TNF-α, tumour necrosis factor

INTRODUCTION

The pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) plays a crucial role in the regulation of the cytokine network during the

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development of arthritis (reviewed in [1-3]). Patients with inflammatory or autoimmune rheumatic diseases have increased levels of circulating TNF-α[4] and transgenic mice overexpressing this cytokine develop arthritis spontaneously at about four weeks of age.[5] Recently, we have shown that the high levels of plasma TNF-α in rheumatoid arthritis patients primes their neutrophils and monocytes so that on stimulation they produce substantially more reactive oxygen species (ROS) than control phagocytes.[6-8] Therefore, one way in which increased levels of TNF-α may contribute to the development of rheumatoid arthritis is by stimulating ROS production by phagocytic NADPH oxidases.

Mitochondria are another major source of ROS in biological systems[9] and about 2–4% of the oxygen consumed by mitochondria is converted to superoxide as a by-product of normal respiration.[10] In pathological situations, such as during ischaemia-reperfusion and exposure to toxins, mitochondrial superoxide production increases substantially.[11] This increase in mitochondrial oxidative stress causes mitochondrial dysfunction and cell damage[11] and may contribute to human diseases including neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases and amyotrophic lateral sclerosis, stroke, epilepsy, ageing and the AIDS dementia complex.[11-13]

Mitochondrial superoxide production by the respiratory chain is stimulated by TNF-α via an unknown mechanism.[9,14] The increased mitochondrial ROS production in rheumatoid arthritis may be caused by elevated plasma TNF-α, and this may contribute to the pathology of this disease. Supporting this, multiple deletions of mitochondrial DNA have been detected in patients with polymyalgia rheumatica, an inflammatory disease with similarities to rheumatoid arthritis.[15]

To measure mitochondrial radical production within intact cells we used the chemiluminescence of lucigenin (bis-*N*-methylacrindinium nitrate).[16] This compound easily permeates membranes and, because of its positive charge, accumulates into the negatively charged mitochondrial matrix driven by the membrane potential.[17] Lucigenin reacts specifically with superoxide to form a chemiluminescent product which can be detected in a luminometer. Therefore, lucigenin is a relatively specific probe for mitochondrial superoxide production within intact cells.[16,17]

The role of mitochondrial oxidative stress in rheumatoid arthritis (RA) has not been investigated. Our study shows that mitochondrial ROS production increases more than five-fold in whole blood and monocytes, but not in neutrophils from patients with RA. This increase in mitochondrial ROS production correlates with an increase in plasma levels of TNF-α. These data suggest that mitochondrial oxidative stress may contribute to the pathogenesis of rheumatic diseases.

MATERIALS AND METHODS

Study cohort

Thirty-five patients with clearly diagnosed rheumatoid arthritis[18] were recruited from the rheumatology unit at Charité hospital, Berlin. Thirty age and sex-matched healthy colleagues on the laboratory and hospital staff served as healthy controls. Thirty-two patients with non-rheumatic internal diseases (IntD) served as disease controls. Of these, 6 had autoimmune hepatitis, 7 neoplasms of various organs, 5 obstructive lung diseases, 5 inflammatory gastritis, cholongitis or abscesses, 4 heart diseases, 2 diabete s mellitus and 3 with *Neisseria gonorrhoe* infections.

Chemicals

RPMI 1640 without phenol red was obtained from Gibco, Paisley, Scotland. Unless indicated otherwise all other reagents were purchased from Sigma-Aldrich.

Monocyte and neutrophil isolation

Blood was drawn by venipuncture and collected in polystyrene tubes coated with 10 mM EDTA.

For determination of whole blood chemiluminescence the blood was stored at room temperature and used without further treatment. For preparation of lymphocytes and neutrophils blood was diluted with an equal volume of RPMI 1640 which did not contain phenol red. Lymphocytes and neutrophils were separated by Histopaque 1119/1077 density gradient centrifugation (700 × *g* for 30 min at 25°C). Contaminating erythrocytes were lysed with ice-cold 0.155 M NH_4Cl/0.01 M $KHCO_3$/0.1 mM EDTA, pH 7.4, and the cells were then washed twice with RPMI 1640. Monocytes were separated from lymphocytes by adherence to plastic Petri dishes for 90 min at 37°C in an atmosphere of 5% CO_2/95% air. Both neutrophils and monocytes were counted using a haemocytometer and diluted with RPMI 1640 to 1×10^6 cells/ml and stored at room temperature. Viability was always greater than 95% by trypan blue exclusion. All experiments were initiated within two hours of bleeding.

Lucigenin-derived chemiluminescence of isolated monocytes, neutrophils and whole blood

Neutrophils or monocytes (2×10^5 cells), or 100 µl EDTA-treated whole blood containing 2.5–3.0 × 10^6 neutrophils and 2.2–3.4 × 10^5 monocytes per ml were added to 600 µl RPMI 1640 medium in a 5 ml plastic tube (Sarstedt, Germany). Lucigenin (100 µl) was added to the mixture from a 1 mM stock solution in RPMI 1640 to give a final concentration of 100 µM. A diphenylene iodoniumchloride (DPI) stock solution (50 µM) was prepared by dissolving DPI in a minimal volume of dimethylsulfoxide (DMSO) which was then diluted in RPMI 1640. This DPI stock (100 µl) was added to the mixture to give a final concentration of 5 µM. The chemiluminescence was measured in a Berthold 953 luminometer (Wildbad, Germany) at 37°C for 1 h and integrated.[20,21] The respiratory chain inhibitors rotenone, antimycin A and oligomycin were dissolved in DMSO and diluted with RPMI 1640 to give a DMSO concentration of 0.05% (v/v) in the stock solutions. Stock solutions of KCN were prepared in RPMI 1640.

Tumor necrosis factor alpha

The concentration of TNF-α in patients' plasma was determined using an alkaline phosphatase-linked immunosorbent assay kit (Immunotech, Marseille, France) in accordance with the suppliers recommendations. Plasma was obtained by centrifugation of EDTA-anticoagulated blood for 15 min at 1000 × *g* at 25°C. Duplicate plasma samples were read within 2 h of collection and compared to a calibration curve obtained with human recombinant TNF-α.

Statistical analysis

All data were analysed with Mann-Whitney's nonparametric test using the *Instat 2.01* program (GraphPad, San Diego, CA) for Apple/Macintosh and are presented as means ± standard deviations (SD). $P<0.05$ was considered significant.

RESULTS

Lucigenin-derived chemiluminescence of whole blood

Thirty-five samples of EDTA-anticoagulated blood from patients with rheumatoid arthritis were examined for their ability to generate lucigenin chemiluminescence as an indication of their rate of production of ROS (Figure 1A). These were compared with thirty age- and sex-matched blood samples from healthy controls and thirty two blood samples from patients with non- rheumatic diseases. Healthy controls showed an average chemiluminescence of 1.0 ± 0.6 × 10^6 cph. In blood from patients with rheumatoid arthritis the chemiluminescence was increased more than 5-fold to 5.5 ± 3.5 × 10^6 cph, which was significantly greater than whole blood chemiluminescence from both healthy and

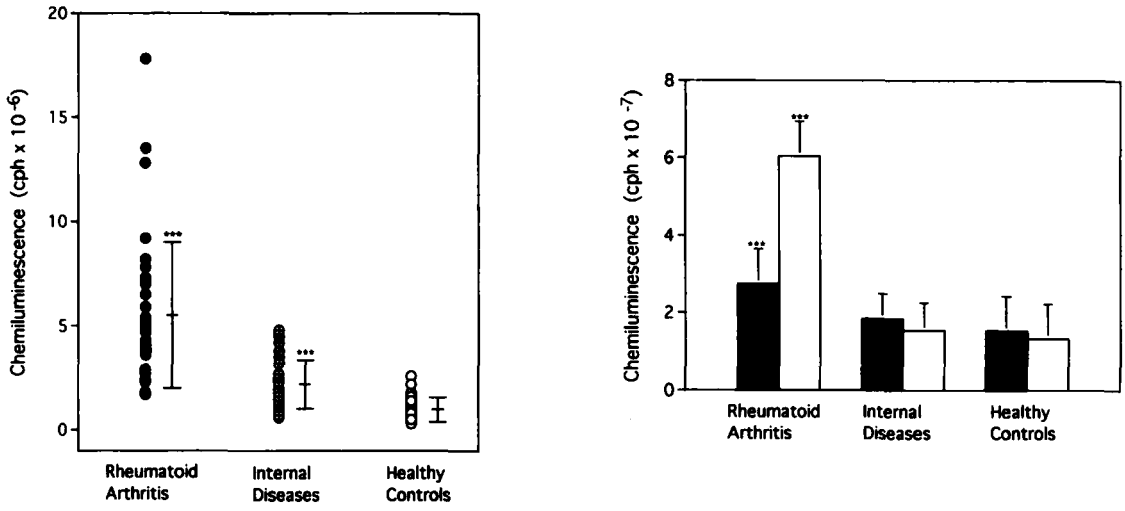

FIGURE 1 Lucigenin-derived chemiluminescence of (A) whole blood and (B) neutrophils and monocytes from patients with rheumatoid arthritis and from control subjects. (A) Blood samples from patients with rheumatoid arthritis (n = 35), healthy controls (n = 30), and subjects with non-rheumatic diseases (n = 32) were examined for their ability to generate chemiluminescence. Duplicate samples were read within two hours of collection and compared to both healthy and non-rheumatic disease controls. The data are presented as means ± standard deviations. (B) The lucigenin chemiluminescence of neutrophils and monocytes isolated from blood from patients with rheumatoid arthritis, healthy controls, and subjects with non-rheumatic diseases was recorded. Each sample was measured in duplicate and the data are presented as means ± standard deviation. Data from experiments with monocytes are shown by open bars and data for neutrophils are shown by closed bars. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, $p>0.05$ not significant (ns).

non-rheumatic disease controls ($p<0.0001$; Figure 1A). The chemiluminescence of non-rheumatic disease controls was $2.2 \pm 1.2 \times 10^6$ cph, which was also significantly greater than blood from healthy controls ($p<0.0001$), but substantially less than the chemiluminescence of blood from patients with rheumatoid arthritis.

Lucigenin-derived chemiluminescence of neutrophils and monocytes

To determine the source of the chemiluminescence, neutrophils and monocytes were separated from blood (Figure 1B). The increased chemiluminescence seen in whole blood from patients with rheumatoid arthritis was also seen in isolated monocytes and neutrophils. For isolated monocytes from healthy individuals the chemiluminescence was $1.3 \pm 1.1 \times 10^7$ cph, which increased 4.6-fold to $6.0 \pm 1.2 \times 10^7$ cph in monocytes of RA patients and was significantly greater than the chemiluminescence of monocytes from healthy controls ($p<0.0001$). This increase in chemiluminescence is similar to that found in unseparated blood (Figure 1A). The value for the chemiluminescence of monocytes from non-rheumatic disease controls was $1.5 \pm 0.9 \times 10^7$ cph, which was not significantly higher than the chemiluminescence of healthy controls. The chemiluminescence of RA patients was also significantly greater than that of monocytes from non-rheumatic disease controls ($p<0.0001$). Isolated neutrophils gave chemiluminescence of $2.7 \pm 0.9 \times 10^7$ cph, which was 1.8-fold greater and significantly different from the chemiluminescence of healthy controls, which was $1.5 \pm 0.9 \times 10^7$ cph ($p<0.0001$). The chemiluminescence of non-rheumatic disease controls was $1.8 \pm 0.7 \times 10^7$ cph, which was significantly lower than that obtained from patients with rheumatoid arthritis but was not significantly different from healthy control subjects ($p = 0.125$).

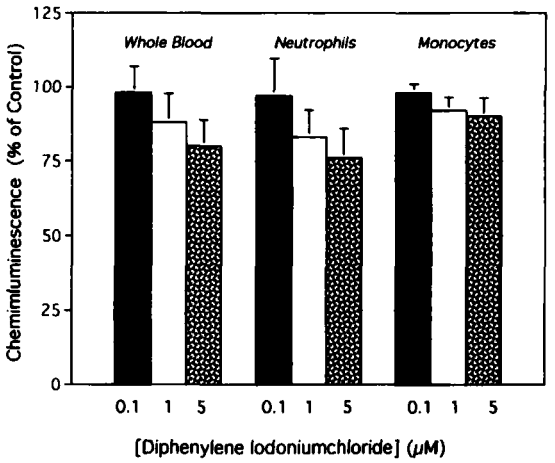

FIGURE 2 Inhibition of lucigenin chemiluminescence by diphenylene iodonium chloride (DPI). Lucigenin-derived chemiluminescence was measured in whole blood, neutrophils and monocytes isolated from 35 RA-patients. The samples were incubated with various concentrations of DPI and the chemiluminescence recorded as described in the materials and methods section. Samples were assayed in duplicate and data are presented as means ± standard deviations.

Decrease of chemiluminescence by inhibition of NADPH oxidase

Whole blood, neutrophils or monocytes from RA-patients were incubated with increasing concentrations of the NADPH oxidase inhibitor DPI to eliminate superoxide production by this enzyme[19] (Figure 2).

Treatment with DPI eliminated up to 20% of the chemiluminescence of whole blood (Figure 2). This inhibition was greater than that found in whole blood from controls (data not shown) in agreement with our earlier reports that NADPH oxidase activity is primed by pro-inflammatory cytokines like TNF-α in patients with rheumatic diseases.[6–8] In isolated neutrophils the DPI inhibition of chemiluminescence was similar to that seen in whole blood, indicating a slight activation of the oxidative burst of these cells due to the isolation procedure.[20] In contrast, DPI did not significantly affect the chemiluminescence of isolated monocytes, suggesting that more than 90% of the chemiluminescence produced by monocytes comes from some other source than NADPH oxidase.

Inhibition of chemiluminescence by mitochondrial respiratory chain inhibitors

As lucigenin is known to report mitochondrial ROS production[16,17] it is probable that the chemiluminescence which cannot be inhibited by DPI arises from mitochondria. To test this possibility whole blood, monocytes and neutrophils were incubated with mitochondrial inhibitors and the effect of these inhibitors on lucigenin chemiluminescence was measured (Figure 3). The inhibitors used were rotenone, an inhibitor of complex I, antimycin A, an inhibitor of complex III, KCN an inhibitor of complex IV and oligomycin, an inhibitor of F_0F_1-ATP synthase.

Incubation of whole blood from RA patients with the respiratory chain inhibitors rotenone, antimycin and KCN decreased the lucigenin chemiluminescence, suggesting a requirement for functional mitochondria in lucigenin chemiluminescence (Figure 3A). In contrast, oligomycin, which inhibits mitochondrial ATP synthesis but does not directly affect the respiratory chain, did not affect chemiluminescence significantly. When this experiment was repeated with monocytes isolated from RA patients the trend of decreasing chemiluminescence in the presence of respiratory chain inhibitors was even more evident than in whole blood (Figure 3B). In monocytes increasing concentrations of respiratory chain inhibitors caused a dose dependent inhibition of chemiluminescence. The maximum inhibition of chemiluminescence (97%) occurred when the complex III inhibitor antimycin A was used. In contrast to the results obtained with the respiratory inhibitors, but consistent with those obtained in whole blood, inhibition of F_0F_1 ATP synthase by oligomycin resulted in only a minor decrease in chemiluminescence. When these experiments were repeated using neutrophils the decrease of chemiluminescence caused by both respiratory inhibitors and oligomycin was small, suggesting that most of the

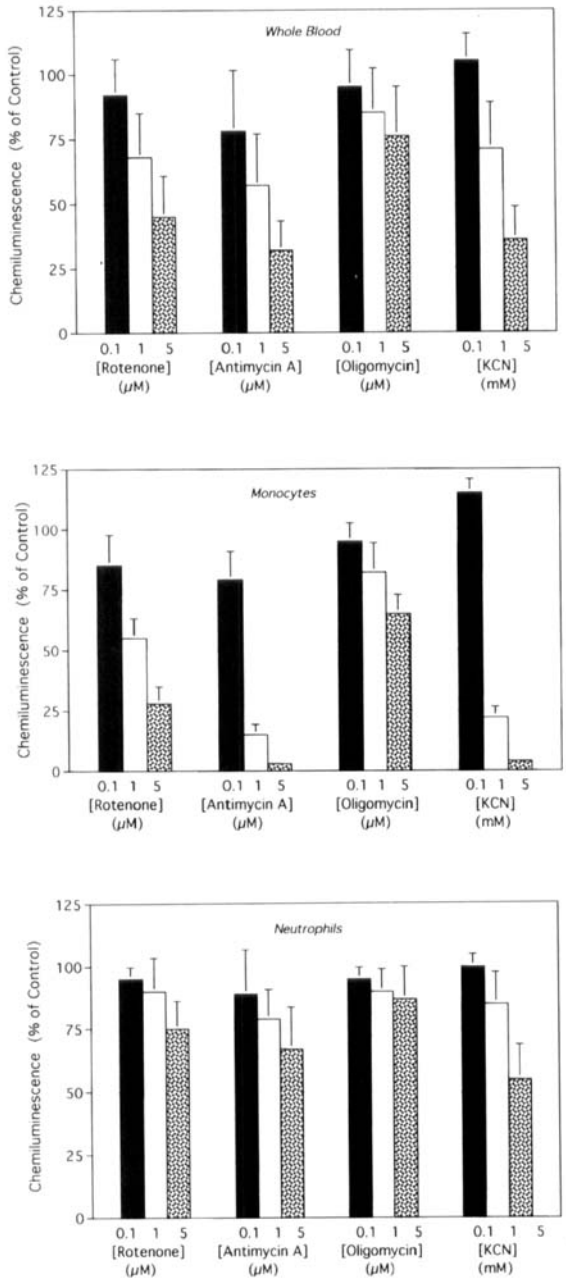

FIGURE 3 Inhibition of lucigenin chemiluminescence by mitochondrial inhibitors in (A) whole blood, (B) monocytes and (C) neutrophils isolated form patients with rheumatoid arthritis. Whole blood, monocytes or neutrophils from RA-patients were incubated with different concentrations of various mitochondrial inhibitors and the lucigenin-derived chemiluminescence measured as described in the materials and methods section. Samples were measured in duplicate and the data are presented as means ± standard deviations.

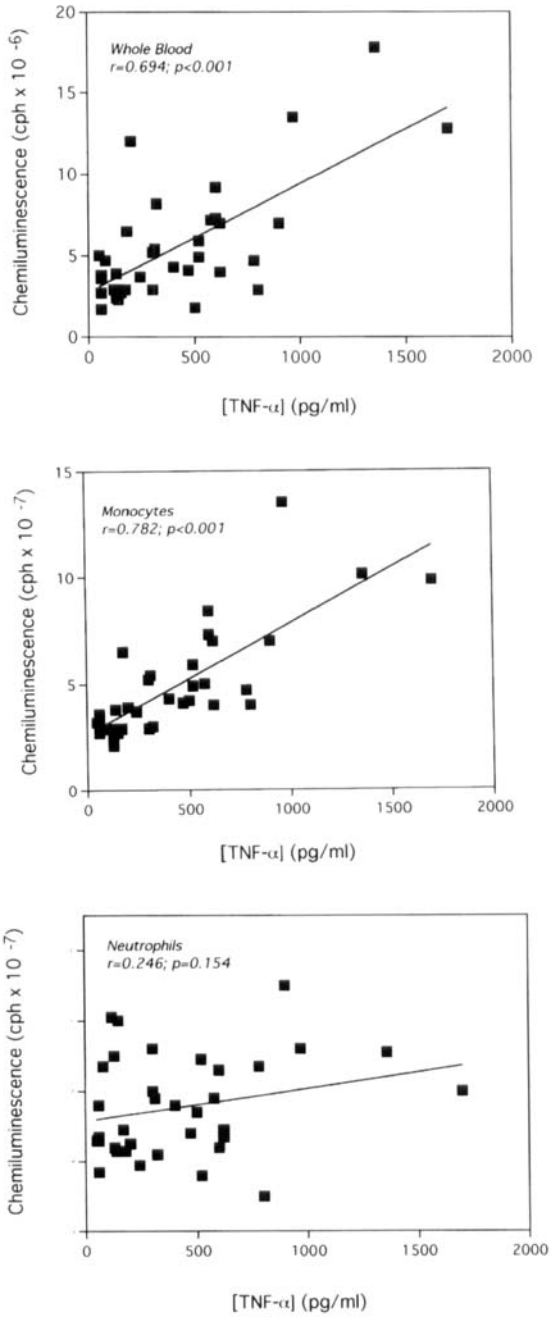

FIGURE 4 Correlation of lucigenin chemiluminescence in whole blood (A), monocytes (B) and neutrophils (C) with plasma levels of tumor necrosis factor alpha. Lucigenin chemiluminescence of whole blood, monocytes and neutrophils from RA-patients was measured as described in the legend to Figure 3. The chemiluminescence was plotted against the concentration of TNF-α in plasma from RA-patients. Pearson's correlation coefficient (r) was used for the linear correlation of data. $P<0.05$ was considered significant.

inhibition of chemiluminescence seen in whole blood was due to inhibition of monocyte ROS production.

Correlation of lucigenin chemiluminescence with plasma concentration of TNF-α

The concentration of TNF-α in plasma from RA-patients was measured by an immunosorbent assay. As has been shown previously the concentration of TNF-α increased substantially in patients with rheumatoid arthritis.[4,8] In this study the plasma concentrations of TNF-α for RA-patients were in the range of 75–1700 pg/ml with a mean value of 430 ± 380 pg/ml. In most healthy control subjects the concentration of TNF-α was less than 25 pg/ml, the detection limit of the assay. To determine whether there was any correlation between the elevated TNF-α concentrations and lucigenin chemiluminescence, the chemiluminescence was plotted against the concentration of TNF-α (Figure 4). For both whole blood (Figure 4A) and monocytes (Figure 4B) there was a positive and significant correlation between the concentration of TNF-α and lucigenin chemiluminescence (r values of 0.69 and 0.78, respectively; $p<0.001$ in both cases). In contrast, for isolated neutrophils there was no significant correlation between the concentration of TNF-α and chemiluminescence (Figure 4a).

DISCUSSION

Increased oxidative stress plays a significant role in the pathology of rheumatoid arthritis.[6–8,20–23] In this study we used the chemiluminescence of lucigenin to measure the production of ROS in whole blood, and in isolated neutrophils and monocytes, from rheumatoid arthritis patients. Our data show that the ROS production in rheumatoid arthritis patients is more than 5-fold greater than in either healthy control subjects or in patients with non-rheumatic diseases. Monocytes and neutrophils isolated from the blood of rheumatoid arthritis patients showed that monocyte chemiluminescence but not that due to neutrophils increased significantly in rheumatoid arthritis patients. Therefore, much of the increased whole blood chemiluminescence found in rheumatoid arthritis patients is caused by stimulation of monocyte ROS production.

The elevated ROS production by whole blood and monocytes from rheumatoid arthritis patients correlated with increased plasma concentrations of TNF-α, in agreement with earlier studies.[8] Extending this earlier finding we have shown that there was a significant positive correlation between plasma TNF-α concentration and ROS production in both whole blood and monocytes from rheumatoid arthritis patients. A correlation between ROS production and plasma TNF-α is consistent with an earlier study which showed that transgenic mice overexpressing TNF-α develop arthritis spontaneously at about four weeks of age, and that a monoclonal antibody against TNF-α can suppress this arthritis.[5,24] Recently, we showed that thalidomide, an inhibitor of TNF-α signal transduction, prevents the development of collagen type II induced arthritis in mice.[25] These data indicate that TNF-α plays a key role in the pathogenesis of rheumatic diseases.

Elevated TNF-α production in rheumatoid arthritis patients causes activation of NADPH oxidase. Earlier, we showed that this enzyme is indeed primed by TNF-α, increasing its sensitivity to stimuli that enhance ROS production by NADPH oxidase.[6–8] Such priming of NADPH oxidase may contribute to rheumatoid arthritis pathology *in vivo*. However, the increased ROS production by unstimulated monocytes reported here was insensitive to DPI, an inhibitor of NADPH oxidase.[19] Therefore, we suggest that the ROS production originates from mitochondria, which are an important source of reactive oxygen species *in vivo*.[26] The respiratory chain loses electrons from two sites to react with oxygen forming superoxide.[9] One of these sites is associated with NADH dehydrogenase (complex I) and the other is associated with complex III. Mitochondrial

radical production increases in a number of pathological situations, including ageing, and can be induced by ischaemia-reperfusion and toxins.[11]

The positively charged lucigenin molecule is accumulated several hundred-fold into mitochondria within cells[16] and lucigenin chemiluminescence is an indication of mitochondrial radical production.[17] Therefore, the increase in lucigenin chemiluminescence may be due to an increase in mitochondrial radical production in monocytes from rheumatoid arthritis patients. Respiratory chain inhibitors, which block mitochondrial electron transport and deenergise mitochondria, strongly suppress lucigenin chemiluminescence in whole blood and monocytes from rheumatoid arthritis patients. The decrease in lucigenin chemiluminescence caused by respiratory chain inhibitors is an apparent paradox, as the respiratory inhibitors antimycin and rotenone have been shown to increase mitochondrial radical production.[9] One possibility is that a stimulation of mitochondrial radical production by respiratory inhibitors may also occur in our experiments. However, inhibition of the respiratory chain will also deenergise the mitochondria, releasing lucigenin from the mitochondrial matrix, where it is usually retained by the membrane potential. As the concentration gradient for positively charged lipophilic compounds across the mitochondrial inner membrane may be several hundred-fold, even if the respiratory inhibitors increase mitochondrial radical production, the decrease in concentration of lucigenin in the mitochondrial matrix on depolarisation will cause a reduction in chemiluminescence.

Consistent with this interpretation oligomycin, an inhibitor of mitochondrial F_0F_1-ATPsynthase, does not decrease lucigenin chemiluminescence. Inhibition of ATP synthesis does not deenergise the mitochondria and therefore a high concentration of lucigenin will be retained within the mitochondria. This lack of inhibition of chemiluminescence by oligomycin also indicates that the decrease in lucigenin chemiluminescence is not due to a decrease in cytosolic ATP concentration. The lucigenin chemiluminescence assay described here has considerable potential as a rapid and convenient screen for oxidative stress-related mitochondrial dysfunction in patients with arthritis and other diseases; a major advantage is that large numbers of blood samples can be analysed rapidly.

The mechanism of activation of mitochondrial ROS production is unclear, but as radical production correlates with an increased plasma concentration of TNF-α it is plausible that TNF-α stimulates mitochondrial ROS production. Consistent with this interpretation TNF-α induces mitochondrial radical production by an unknown mechanism.[9,14,27] Increased mitochondrial radical production will result in oxidative damage to the cell, and cause mitochondrial calcium efflux and depolarisation.[28] Mitochondrial oxidative stress may contribute to the pathology of rheumatoid arthritis by increasing cytosolic oxidative stress, subsequently activating the transcription factor NF-κB, which stimulates the further expression of TNF-α.[29–31] We are currently investigating the molecular mechanisms by which TNF-α enhances mitochondrial oxidative stress in experimental models of arthritis, and determining whether mitochondrial oxidative stress increases the expression of TNF-α.

Acknowledgments

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