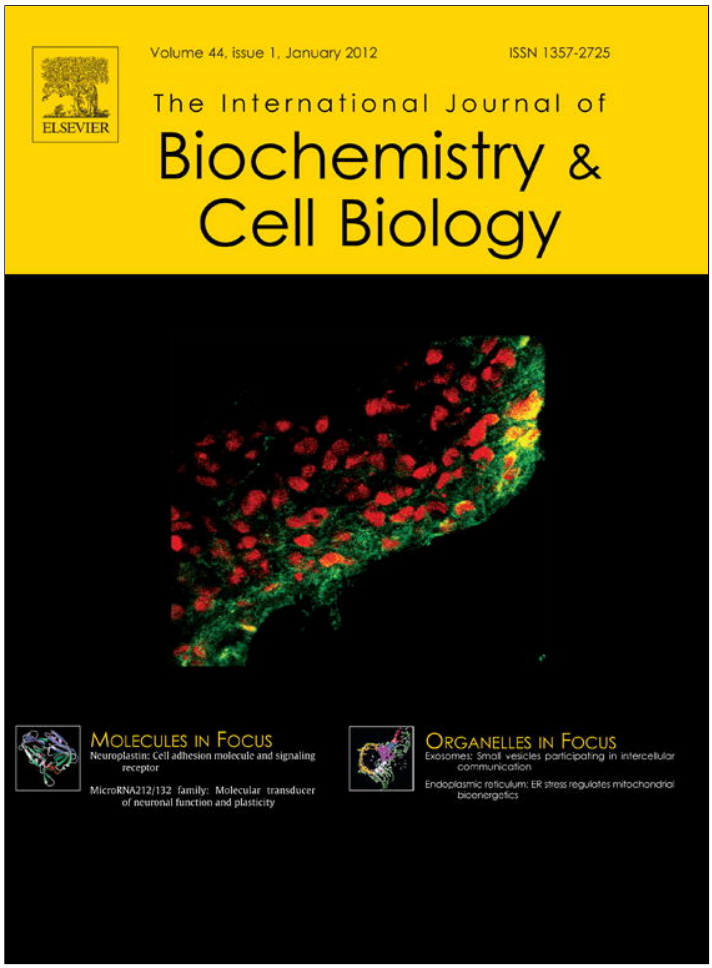

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TNFα affects energy metabolism and stimulates biogenesis of mitochondria in EA.hy926 endothelial cells

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ABSTRACT

Mitochondrial response of EA.hy926 endothelial cells to tumour necrosis factor alpha (TNFα) was investigated. It was confirmed that TNFα stimulates reactive oxygen species (ROS) generation and increases intercellular adhesion molecule-1 (ICAM-1) level. These changes were paralleled by elevated oxygen consumption, slightly raised total mitochondrial mass and increased manganese superoxide dismutase (Mn-SOD) and uncoupling protein 2 (UCP2) content. They also correlated with a rise of mitochondrial transcription factor 1 (TFAM), nuclear respiratory factor-1 (NRF-1) and peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α, which are involved in regulation of mitochondrial biogenesis and an elevated level of selected respiratory chain proteins. Thus, the apparent stimulatory effect of TNFα on mitochondrial metabolism probably reflects an increased amount of mitochondria rather than activation of biochemical processes per se, although the latter cannot be excluded definitely. These observations are similar to those described for cardiac muscle cells challenged with bacterial lipopolysaccharide (LPS), in which mitochondrial biogenesis was postulated. Stimulation of mitochondrial biogenesis could be a mechanism activated to prevent TNFα-induced cell death.

1. Introduction

The vascular endothelium plays an important role in smooth muscle relaxation, vasodilation and eventually regulation of blood flow. Nitric oxide produced and released upon stimulation of endothelial cells as well as the finely controlled amount of ROS which serve as signalling molecules necessary for proper endothelial functioning are of a crucial importance in these processes. On the other hand, under various pathological conditions (e.g. hyperglycaemia) endothelial cells undergo proinflammatory stimulation that may result in inflammation, severe damage to the vascular endothelium, and eventually blood vessels-related pathology (for rev. see Sprague and Khali, 2009).

A critical role in the initiation and acceleration of inflammatory reaction is played by TNFα which belongs to endothelial cell-activating proinflammatory and cytotoxic cytokines. TNFα has been found to affect endothelial cells by activating one of its specific receptors, Tumour Necrosis Factor Receptor (TNFR) 1 or TNRF-2 although TNFR-1 seems more important in the general TNFα-induced signal transduction, while TNFR-2 is mostly active in the lymphoid system (for rev. see Wajant et al., 2003). TNFα may activate a number of signalling pathways in endothelial cells, either prosurvival and inflammatory ones or those inducing apoptotic cell death. In the former case activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPK) plays a prominent role and results in stimulated expression of genes encoding adhesion molecules (E-selectin, ICAM-1 and vascular cell adhesion protein-1). Additionally it stimulates the synthesis and secretion of other cytokines (interleukin-8 and monocyte chemotactic protein-1). These effects result in leukocyte recruitment and adhesion, development of vascular inflammation and increased cell infiltration (Carlos et al., 1990). TNFα affects (stimulates or reduces) expression of genes encoding nitric oxide synthase (NOS) isoforms, and thus impairs endothelium-regulated vasodilation of blood vessels. In human umbilical vein endothelial cells, to which the EA.hy926 cell line is closely related, TNFα reduces endothelial (eNOS) and constitutive NOS (cNOS) levels but it activates the expression of inducible NOS (iNOS)-encoding gene. The latter effect probably partially depends on NF-κB (Lee et al., 2004; for rev. see Zhang et al., 2009). Stimulation of the NF-κB-dependent pathway which, is the

Abbreviations: Cu/Zn-SOD (SOD1), copper/zinc superoxide dismutase; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; IKK, IkappaB kinase; IκB-α, inhibitor κB-α; iNOS, inducible NOS isoform; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinases; Mn-SOD (SOD2), manganese superoxide dismutase; NADPH-oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; NF-κB, nuclear factor κB; NO, nitric oxide; NOS, nitric oxide synthase; NRF-1, nuclear respiratory factor-1; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; ROS, reactive oxygen species; TFAM, mitochondrial transcription factor 1; TNFα, tumour necrosis factor alpha; UCP 2, uncoupling protein 2.

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most important response of cells to TNFα, requires activation of IkappaB kinase (IKK) which phosphorylates and thereby inactivates inhibitor κB-α (IκB-α). This in turn results in an activation of NF-κB which influences expression of numerous genes involved in regulation of immunity, cell survival and differentiation (Kempe et al., 2005). Apart from the classical NF-κB pathway another mode of signal transduction following IKK activation has also been considered. Activated IKK located in the cytosol has been postulated to act as a regulator of mitochondrial biogenesis, as shown in skeletal muscle cells (Bakkar et al., 2008).

Another TNFα-induced scenario is based on activation of a proapoptotic signalling pathway in which excessive ROS generation, activation of caspases and mitochondria-dependent proapoptotic events eventually lead to cell death. The TNFα-induced apoptosis is prevented by simultaneous activation of NF-κB, thus a final balance between the prosurvival and proapoptotic pathways in TNFα-stimulated cells determines their fate (Rath and Aggarwal, 1999).

Endothelial ROS formation is mainly attributed to the action of cytosolic nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase and mitochondria, albeit their relative input to TNFα-evoked cell death is still disputable. It is beyond any doubt, however that the proapoptotic stimulation of cells by TNFα results in mitochondrial impairment and enhanced ROS formation that is harmful to cells and thus is recognized as a proapoptotic factor (Kim et al., 2010). ROS are generated in mitochondria as a by-product of the respiratory chain activity with an intensity dependent on numerous intra- and extracellular factors. Under physiological conditions the ROS originating from mitochondria serve as signalling molecules participating in important cellular processes including mitochondrial biogenesis [For rev. see Bae et al., 2011; Di Lisa et al., 2009]. TNFα may affect mitochondrial functioning and thereby cause abnormal mitochondrial ROS production. In fact, various cells exposed to TNFα exhibited inhibition of respiratory chain activity, a disturbance known to lead to enhanced superoxide production (Stadler et al., 1992; Corda et al., 2001).

The aim of the present work was to investigate TNFα-evoked changes in mitochondrial metabolism in EA.hy926 cells. We found that exposition of EA.hy926 cells to TNFα induces changes suggesting an increased mitochondrial biogenesis rather than an inhibition of cellular energy metabolism. Such conclusion is similar to that drawn from experiments on neonatal rat cardiomyocytes treated with bacterial LPS, where the authors also found stimulation of mitochondrial biogenesis acting as an anti-apoptotic event (Hickson-Bick et al., 2008).

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells EA.hy926, passage 1–12 (kindly provided by Dr. Antoni Wrzosek) were grown in low glucose (1 mg/ml) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (Gibco Invitrogen), antibiotics (penicillin 100 units/ml plus streptomycin 50 μg/ml, Sigma) in a humidified atmosphere of 5% CO_2/95% air at 37 °C. The growth medium was changed every two days. After attaining confluence the cells were treated with TNFα (5 ng/ml) for 6 h.

2.2. Oxygen consumption

Cellular respiration was measured polarographically at 37 °C using an OROBOROS Oxygraph-2k (OROBOROS® INSTRUMENTS GmbH, Austria). Confluent monolayers (grown in tissue culture dishes of 10 cm in diameter) were trypsinized and the cell suspension was diluted with the same volume of culture medium, centrifuged (at 150 × *g* for 3 min) and resuspended in PBS prewarmed to 37 °C to a final protein concentration of approximately 0.5 mg/ml. Oxygen consumption was measured in the presence of substrates and inhibitors as indicated in figure legends. The respiration rate was expressed as the decrease in oxygen concentration per second per mg protein.

2.3. Mitochondrial membrane potential

Mitochondrial membrane potential was measured fluorimetrically with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide; Molecular Probes, Invitrogen) according to the method published by Cossarizza et al. (1993). Confluent cells grown on 24-well culture plates were incubated in the culture medium with 5 μM JC-1 dye at 37 °C in the dark for 15 min. Then, the cells were rinsed three times first with the culture medium then with PBS. Finally, 0.5 ml PBS was added to each well. To achieve complete dissipation of the mitochondrial membrane potential the cells were incubated with 2 μg/ml of valinomycin and 5 μM CCCP prior to staining with JC-1 dye.

JC-1 fluorescence was measured with the use of a laser scanning cytometer (iCYS CompuCyte) equipped with argon laser (excitation 488 nm). Data were presented as the ratio of orange (highly energized mitochondria) to green (deenergized mitochondria) fluorescence.

2.4. Reactive oxygen species formation

Reactive oxygen species were measured fluorimetrically with carboxy-2′,7′-difluorodihydrofluorescein – $DFFH_2$-DA (Molecular Probes, Invitrogen). Cells grown as a confluent monolayer on 12-well plates were stained with 10 μM probe in PBS at 37 °C in the dark for 30 min. Then, the fluorescence was measured using a SpectraMax micro plate reader (Molecular Devices) at 485 nm and 520 nm for excitation and emission, respectively. Results were expressed per mg protein.

2.5. NO production

Nitric oxide generation was detected fluorimetrically with nitric oxide indicator – DAF-FM diacetate – (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate). Cells grown as a confluent monolayer on 12-well plates were stained with 1 μM probe in PBS at 37 °C in the dark for 1 h. Then, the cells were rinsed with PBS and incubated for 15 min to allow complete deestrification of the probe. Fluorescence was measured using a SpectraMax micro plate reader (Molecular Devices) at 495 nm and 515 nm wavelength for excitation and emission, respectively. Results were expressed per mg protein (López-Figueroa et al., 2000; Berkels et al., 2000).

2.6. Mitochondrial mass

Total mitochondrial mass (amount of mitochondria) was measured fluorimetrically with Mitotracker Green (Molecular Probes, Invitrogen). Confluent cells grown on 24-well plates were stained with the dye at 100 nM, diluted in the culture medium, for 20 min at 37 °C. Next, the cells were rinsed three times with the culture medium and then with PBS. Finally, 0.5 ml PBS was added to each well. Fluorescence was measured using laser scanning cytometer iCYS equipped with argon laser (excitation 488 nm). Total fluorescence was calculated per mg protein.

2.7. Cell lysis, electrophoresis and Western blot

Cell monolayers were rinsed twice with cold (4 °C) PBS and drained carefully. Then, ice-cold lysis buffer (Cell Signalling Technology) supplemented with protease inhibitors cocktail (Roche), sodium fluoride (NaF, 10 mM) and phenylmethylsulphonyl fluoride (PMSF, 1 mM) was added (300 μl per dish). Cells were scrapped and forced through a thin needle 2–3 times. The lysates were incubated for 20 min on ice and centrifuged at 15,000 × *g* at 4 °C for 20 min. Supernatants were transferred into fresh tubes. Protein concentration was measured using a modified Lowry protein assay kit (Thermo Scientific).

An appropriate amount of the sample buffer was added to each probe and the samples were boiled at 95 °C for 5 min. After cooling, they were stored at −20 °C. Samples for mitochondrial complexes electrophoresis were prepared in the same way with the exception of boiling.

Polyacrylamide gel electrophoresis (PAGE) under denaturing conditions in the presence of 0.1% sodium dodecyl sulphate (SDS) was performed. To detect selected proteins specific primary antibodies were used. All secondary antibodies conjugated with horseradish peroxidase (HRP) were obtained from Abcam. Chemiluminescent substrate Luminata Classic or Crescendo (Millipore) were used for HRP detection. The amount of selected proteins was calculated densitometrically and expressed in relation to β-actin (Monoclonal Anti-β-Actin-Peroxidase antibody, Sigma). The following antibodies against proteins of interest were used: ICAM-1 (Santa Cruz), UCP2 (Abcam), SOD1 (Santa Cruz), Mn-SOD (BD Transduction Laboratories), NRF-1 (Santa Cruz), PGC-1α (Santa Cruz), TFAM (GeneWay Biotech), MitoProfile Total OXPHOS Human WB Antibody Coctail (MitoSciences).

2.8. Immunocytochemistry

For mitochondrial organization, cells grown on coverslips were loaded with 300 nM MitoTracker CMX Ros (Invitrogen, San Diego, CA, USA) for 20 min at room temperature. After rinsing with the growth medium the cells were fixed with 4% paraformaldehyde, rinsed with PBS containing 10% FBS and sealed in Glycergel Mounting Medium (DakoCytomation).

For cytoskeleton immunostaining, cells were incubated for 1 h with FITC-labelled phalloidin (Molecular Probes) in PBS/10% FBS/0.2% saponin, and 30 nM 4,6-diamidine-2-phenylindole diacetate (DAPI, Sigma). Confocal fluorescence microscopy was carried out using a Leica TCS SP5 IL Spectral Confocal and Multiphoton Microscope.

2.9. Expression of results

Data shown are means ± S.D. for the number of separate experiments indicated in the figure legends. The statistical significant differences were calculated using Student's *t*-test.

3. Results

3.1. Inflammatory markers, ROS, NO and superoxide dismutase in EA.hy926 cells upon TNFα treatment

Previously it was shown by other authors that exposition of various cell types including endothelial cells to TNFα affected some metabolic parameters, such as ROS generation or mitochondrial superoxide dismutase level, which suggested changes in mitochondrial metabolism. However, the available data come from experiments on cells derived from various sources or performed under different conditions, particularly regarding TNFα concentration and duration of cell exposition to it (Stadler et al., 1992; Szczygieł et al., 2012).

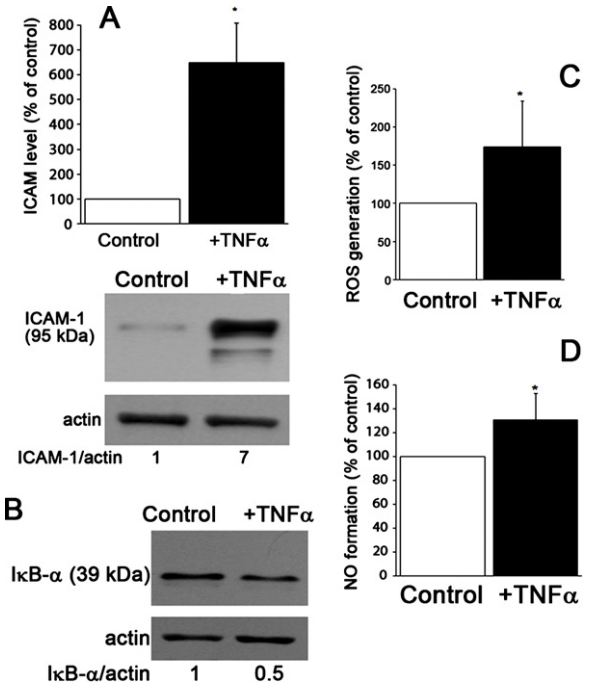

Fig. 1. Effect of TNFα on ICAM-1 and NF-κB protein level and nitric oxide formation. (A) ICAM-1 protein level relative to β-actin, expressed as a percentage of control (non-treated with TNFα). Data from 4 experiments. $^*p < 0.001$. Western blot shows one typical experiment out of four. (B) IκB-α. Western blot from one experiment out of three (C) ROS formation – percentage of control (non-treated with TNFα). Data from 4 experiments. $^*p < 0.03$. (D) NO formation – percentage of control (non-treated with TNFα). Data collected from 5 experiments. $^*p < 0.02$.

In this paper we employed EA.hy926 cell line as a model of vascular endothelium and focused our interest on mitochondrial response in these cells exposed to TNFα. EA.hy926 cell line was obtained by a fusion of HUVEC cells with human lung carcinoma cell line A549 (Bouis et al., 2001), thus it is very useful for simplicity of maintaining, although their potential aberrance must also be considered (Barańska et al., 2005). To characterize a well-defined background for further studies on TNFα-evoked effects on mitochondrial function we verified available data concerning cellular response to TNFα under a single set of conditions. In all experiments presented here the cells were exposed to 5 ng/ml TNFα for 6 h. Under such treatment the cell viability seemed unaffected, as judged by PI staining of live or fixed cells (not shown), while ICAM-1 protein content (Fig. 1A), ROS production (Fig. 1C) and NO synthesis (Fig. 1D) were substantially increased. In addition, the cellular content of IκB-α was significantly reduced (Fig. 1B), suggesting activation of the NF-κB factor and presumably also the NF-κB-dependent signalling pathway. These results generally confirmed the previously described by other authors effects of TNFα on various types of cells.

Additionally, the TNFα-treated cells exhibited an increased amount of mitochondrial Mn-SOD, but surprisingly, the cytosolic form of superoxide dismutase (Cu/Zn-SOD) was slightly but reproducibly reduced (Fig. 2). Taken together, the observed effects indicate induction of an inflammatory response as well as changes in mitochondrial metabolism in these cells. The increased level of Mn-SOD is in line with the activation of NF-κB which is thought to be involved in regulation of the Mn-SOD gene transcription (Sompol et al., 2006).

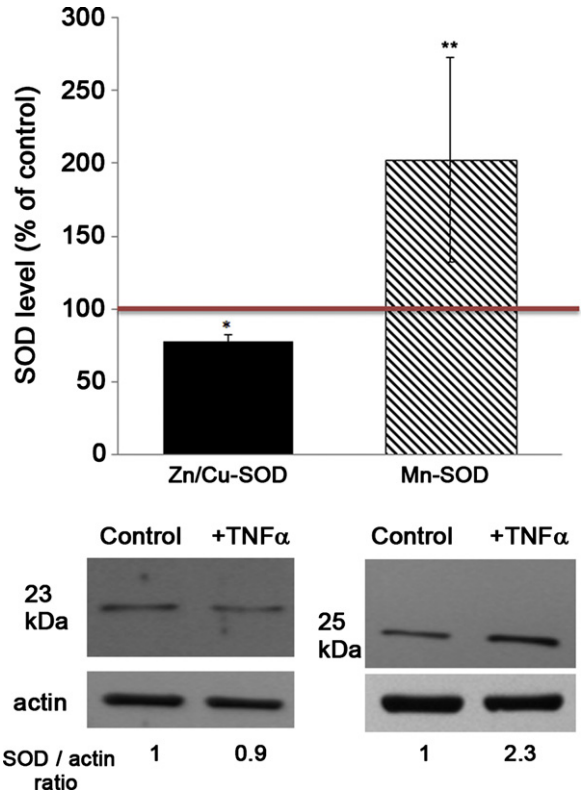

Fig. 2. Effect of TNFα on superoxide dismutases protein level. Data from 3 or 4 experiments for Zn/Cu-SOD and Mn-SOD, respectively. $^{*}p < 0.01$; $^{**}p < 0.03$. Western blot shows one typical experiment out of three for Zn/Cu-SOD (left) or out of four for Mn-SOD (right).

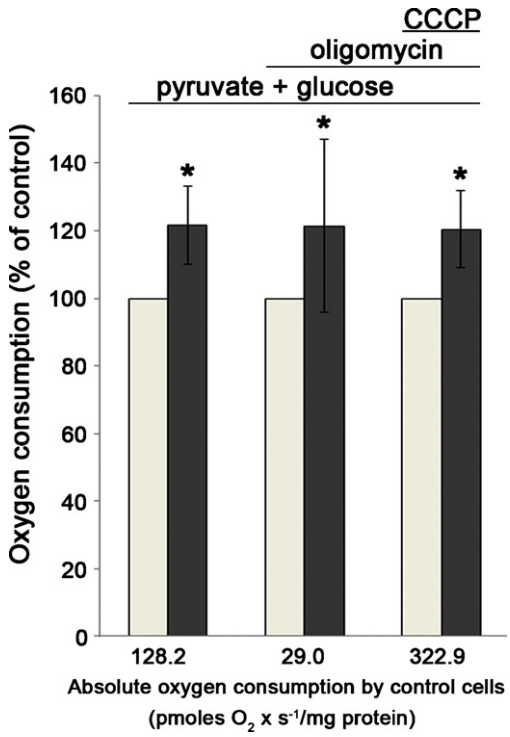

Fig. 3. Oxygen consumption by TNFα-treated cells. Relative increase of oxygen consumption is shown. Bright and dark bars correspond to control (assumed as 100%) and TNFα-treated cells, respectively. Absolute rates of oxygen consumption (pmoles $O_2 \times s^{-1}$/mg protein) were as follows: in the presence of pyruvate + glucose, 128 ± 8; after addition of oligomycin it dropped to 29 ± 5; after subsequent addition of CCCP it raised to 323 ± 18. $n = 5$, $^{*}p < 0.03$. Substrates and inhibitors were sequentially added directly to the incubation chamber as indicated above the bars to final concentrations: pyruvate 1 mM, glucose 5 mM, oligomycin 0.1 μg/ml, CCCP 0.5 μM.

3.2. Oxygen consumption, mitochondrial membrane potential and uncoupling protein

To assess details of the mitochondrial response to TNFα several standard tests were performed. As shown in Fig. 3, oxygen consumption in cells treated with TNFα was considerably enhanced and this effect was observed not only in the presence of respiratory substrates alone but also after addition of oligomycin and a mitochondrial uncoupler (CCCP). The similar extent of stimulation under all conditions tested seems to exclude the possibility that the TNFα-evoked increase in oxygen consumption reflects changes in the coupling state of the mitochondria but rather suggests an increase of the total respiratory capacity, presumably due to more abundant mitochondria in these cells. This result stands in contrast to the previously published data indicating that TNFα exerts an inhibitory effect on the mitochondrial respiratory chain as a mechanism involved in TNFα-induced hepatocyte injury (Stadler et al., 1992). Such a discrepancy could result from different sensitivity to TNFα of the two types of cells studied (primary hepatocytes versus "immortalized" endothelial cells), and/or differences in experimental protocols, including TNFα concentration and duration of cell treatment.

Moreover, the slightly increased (or at least unchanged) mitochondrial membrane potential $\Delta\Psi$ (Fig. 4) convincingly excludes any TNFα-evoked damage to mitochondria. A lack of mitochondrial deenergization despite a substantial enhancement of UCP2 content (Fig. 4) is somewhat confusing as UCP2 protein activity is believed to dissipate the mitochondrial membrane potential (Fink et al., 2002). On the other hand, it is in line with the concept of an increased total amount of mitochondria and not an enhanced mitochondrial metabolic capacity as the main cause of the observed increased oxygen consumption.

Taking together, it is difficult to decide at this stage of the study whether the increase in UCP2 level has an input into the increased oxygen consumption (if so then it should correlate with a decreased mitochondrial membrane potential, which is not the case) or, as suggested here, whether it simply reflects an increase in the total mitochondrial mass.

3.3. Mitochondrial biogenesis and organization

Convincing evidence supporting the latter possibility comes from Western blot analyses revealing a substantial TNFα-induced increase in the levels of PGC-1α, TFAM and NRF-1 (Fig. 5), known activators of mitochondrial biogenesis (Kelly and Scarpulla, 2004). To make things perfectly clear selected respiratory complexes protein level in control and TNFα-treated cells was compared. As shown in Fig. 6A and B an exposition of cells to TNFα resulted in substantially increased amount of components of the mitochondrial respiratory chain complexes II and III. Similar increase was also observed for mitochondrial complex I and IV.

Moreover, laser scanning cytometry with MitoTracker Green dye showed a small but reproducible increase of the total mitochondrial mass upon TNFα treatment of EA.hy926 cells (Fig. 7C). Such an increase of the total mitochondrial mass is in line with all the results presented hitherto (apart from the reduced Cu/Zn-SOD protein level), including the rise of total cellular Mn-SOD protein content. Furthermore, confocal microscopy of mitochondrial network organization revealed mostly perinuclear localization of mitochondria in TNFα-treated cells while in control cells mitochondria were spread throughout the cell (Fig. 7B(c) and (d)). This small effect may be related to a slight change in actin cytoskeleton (Fig. 7B(a) and (b)). Moreover, mitochondria in TNFα-treated

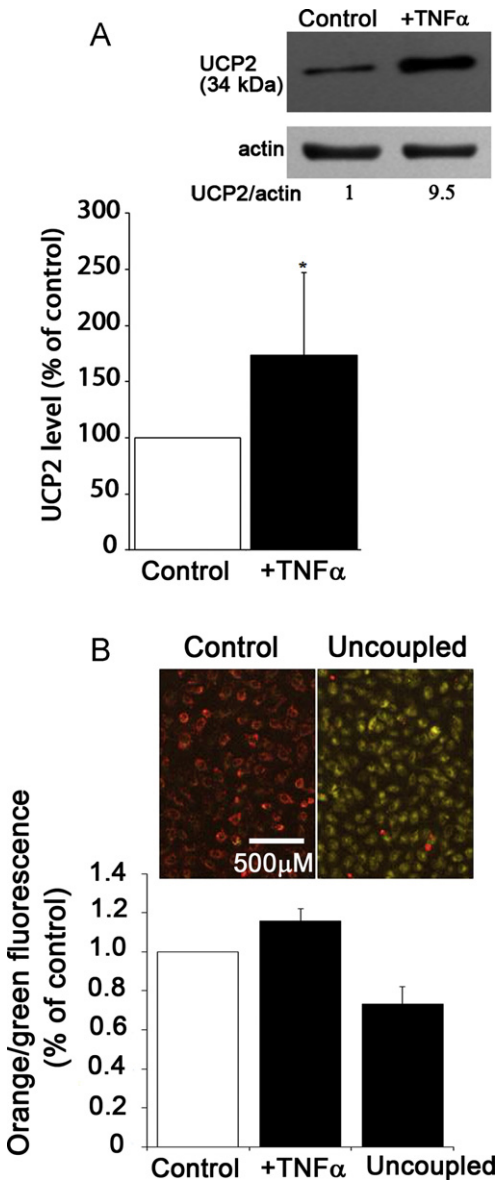

Fig. 4. Effect of TNFα on UCP2 level and mitochondrial membrane potential. (A) UCP2 protein level relative to β-actin, expressed as a percentage of control (non-treated with TNFα). Data from 7 experiments. $^{*}p < 0.05$. Western blot for one typical experiment out of seven. (B) Mitochondrial potential measured with JC-1 probe. Control represents cells non-treated with TNFα (red fluorescence). After mitochondrial uncoupling JC-1 fluorescence switches to green. Below, orange/green fluorescence ratio in cells loaded with JC-1. In control cells it is assumed to be 1. Bar on the right represents orange/green JC-1 fluorescence ratio in control cells treated with CCCP to complete dissipation of mitochondrial potential. Data from 3 experiments. $p < 0.03$ for TNFα; $p < 0.02$ for complete uncoupling.

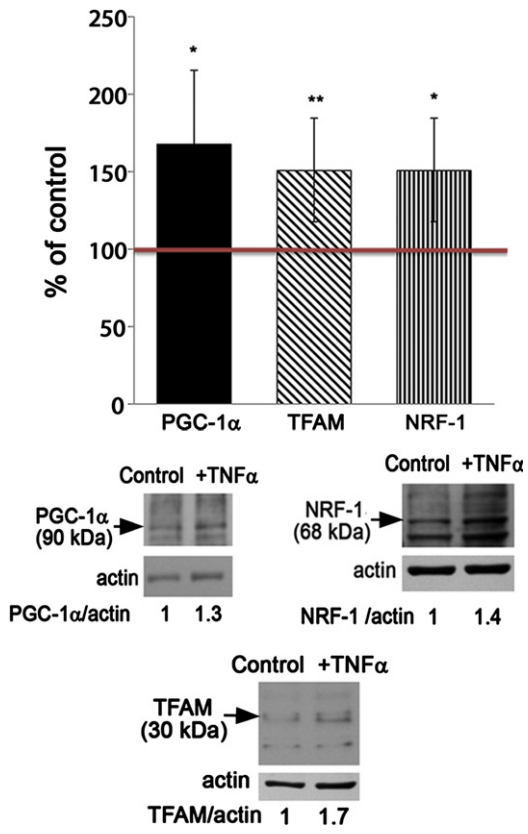

Fig. 5. Effect of TNFα on transcription factors affecting mitochondrial biogenesis. PGC-1α, TFAM and NRF-1 protein level relative to β-actin, expressed as a percentage of control (non-treated with TNFα). Data from 4 experiments. $^{*}p < 0.02$; $^{**}p < 0.03$. Below, Western blots from one typical experiment out of four.

cells tended to form tubular structures (Fig. 7A(b)). These changes could reflect cellular TNFα-induced stress response protecting the mitochondria against autophagosomal degradation (Rambold et al., 2011).

Taking together, the results presented in this paper suggest that stimulation of EA.hy926 cells with TNFα activates multiple processes including induction of inflammatory response, stimulation of antioxidative defence and probably mobilization of mitochondrial biogenesis. The latter seems to be part of a prosurvival stress-related effects which could be an alternative response of cells to an extracellular stimulus potentially leading to cell death.

4. Discussion

TNFα is commonly used to induce inflammatory response in vitro in both primary cell cultures and in immortalized cell lines. The former, being unmodified by any genetic manipulations, seem more appropriate as an experimental material. On the other hand, they are (if from a human source) usually derived from different donors of non-uniform genetic background, which may lead to confusing results that cannot be easily unified. In balance, the immortalized cell lines, despite some reservations, seem advantageous for experimental use (Barańska et al., 2005).

Endothelial cells have been investigated intensively for many years because of their crucial role in the regulation of vascular tone and eventually blood flow. However, for a long time they were commonly recognized as primarily dependent on anaerobic metabolism and consequently endothelial mitochondria were hardly studied (Davidson and Duchen, 2007; Quintero et al., 2006; Culic et al., 1997). It is clear nowadays that in fact mitochondria play an important role in many endothelial processes including intracellular calcium signalling and homeostasis, apoptosis, NO synthesis and ROS generation. In other words, an increased interest in endothelial mitochondria role follows the growing understanding of their numerous functions, which are not directly related to energy metabolism (Davidson and Duchen, 2007).

The inflammatory reaction of the endothelium is a cellular response to extracellular stimuli which appears under various pathological conditions. One of the most prominent markers of inflammation is the adhesive molecule ICAM-1. Its amount is substantially increased in cells stimulated by proinflammatory cytokines and is accompanied by enhanced ROS generation. In this paper both parameters were used to verify the proper response of

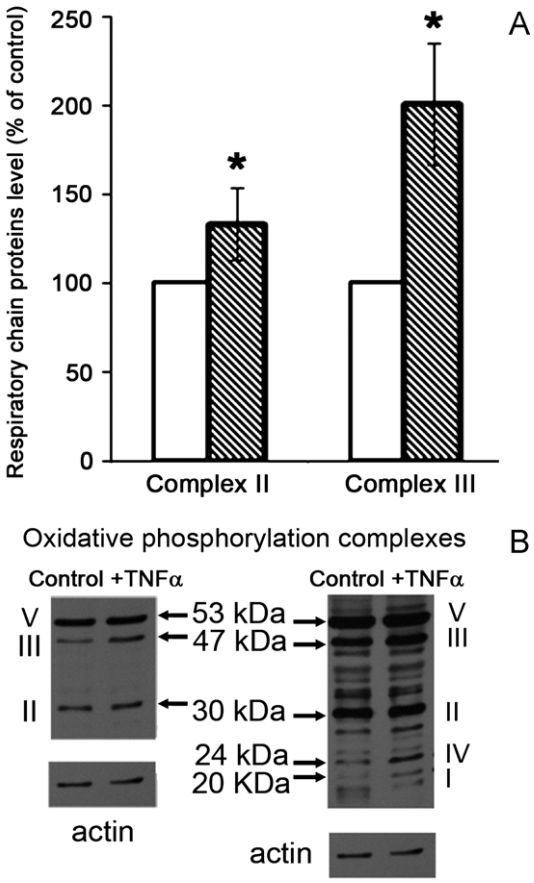

Fig. 6. Effect of TNFα on selected protein components of the mitochondrial respiratory chain. (A) Protein level of respiratory complex II and III components relative to β-actin, expressed as a percentage of control (non-treated with TNFα). Data from 3 experiments. $^{*}p < 0.01$; $^{**}p < 0.05$. (B) For better visualization of particular respiratory proteins the film was exposed twice for shorter (left panel) or longer time to obtain bands of appropriate density.

the cell line studied to TNFα. Although an increased generation of ROS may result not only from aberrant mitochondrial function but also from activation of non-mitochondrial sources, the role of mitochondria in endothelial inflammation must be seriously considered (For rev. see Zhang and Gutterman, 2007).

The TNFα-induced changes in cellular physiology are multifarious and dependent on several factors including cell type, receptor type, cytokine concentration and duration of the treatment (Wajant et al., 2003). Generally, TNFα is recognized as a proinflammatory stimulus activating NF-κB and stimulating expression of anti-apoptotic/prosurvival genes. On the other hand, TNFα may also cause excessive ROS formation and trigger cell death. The balance between these two possibilities seems to depend on the intensity of treatment with TNFα (Rangmani and Sirovich, 2006). That is probably why the incubation of cells with TNFα for six hours used here did not induce any detectable hallmarks of apoptosis (data not shown) despite an increased ROS production (see Fig. 1). On the other hand, the antiapoptotic effect of NF-κB in cells excited with TNFα has been contested in view of the data indicating p38 (MAPK 14), protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) as major players involved in survival pathway activation in bovine aortic endothelial (BAE) cells (Clermont et al., 2003). The cellular response to TNFα is probably cell-type-specific.

The activation of NF-κB expected from the observed IκB-α protein degradation upon treatment of the cells with TNFα could also explain an increase in Mn-SOD protein content (see. Fig. 2), although the link between NF-κB activation and Mn-SOD expression is still disputable. The increased amount of Mn-SOD could also be partially responsible for the resistance of TNFα-stimulated cells to apoptosis (Delhalle et al., 2002). Similarly, the observed activation of NO formation in TNFα-treated cells could also be attributed to NF-κB-dependent activation of iNOS gene expression (Tanimoto et al., 2007; Lee et al., 2004).

Recently, Szczygieł et al. (2012) described a transient inhibition of NO formation in EA.hy926 cells exposed to TNFα followed by substantial stimulation of NO production and then return to the control level approximately at the sixth hour of the treatment. In the present study incubation of cells with TNFα for 6 h resulted in a substantially increased NO formation. This discrepancy probably reflects differences in TNFα concentration applied (10 ng/ml in the mentioned paper versus 5 ng/ml in this study). Those authors also described a major TNFα-induced time-dependent reorganization of the actin cytoskeleton in HMEC cells. Similar changes were observed here (see Fig. 7), although the different cell lines used in the two different studies do not allow a meaningful detailed comparison.

It is well documented that cellular stress is expressed on many levels of cellular organization, with mitochondria playing a prominent role. The stress-induced mitochondrial response depends on the stressor type, duration of its action and cell type. In fact two opposite scenarios may be considered. First, a cellular stress may activate apoptosis, connected with aberrant mitochondrial metabolism and excessive ROS generation, loss of membrane potential, opening of the mitochondrial permeability transition pore and release of selected mitochondrial proteins from the intramembrane space (Kim and Lemasters, 2003). The converse possibility is activation of rescue mechanisms which protect the cell against death and support its survival. In such a scenario the mitochondria have to retain metabolic integrity. In fact, stress-induced stimulation of cells leading to impaired protein synthesis has been shown to result in mitochondrial hyperfusion considered a novel adaptive pro-survival response to proapoptotic stimulation (Tondera et al., 2009). More recently it has been shown that the formation of mitochondrial tubular network protects against autophagosomal degradation (mitophagy) during starvation of mouse embryonic fibroblasts (MEF cells) (Rambold et al., 2011).

Here it is also shown that the reorganization of mitochondrial architecture in EA.hy926 cells exposed to TNFα (see Fig. 7) is accompanied by a substantial elevation of oxidative phosphorylation-related proteins content and less pronounced but reproducible increase in total mitochondrial mass. This may suggests an increase of inner mitochondrial membrane surface and folding. These changes are reflected by higher oxidative capacity (see Fig. 3) and are in line with elevated TFAM protein level and the other transcription factors tested (see Fig. 5). Similar apparent disproportion between an elevation of respiratory chain complexes protein level and a total mitochondrial mass were found in NARP osteosarcoma cybrids (Wojewoda et al., 2011).

Considering that stimulation of neonatal rat cardiomyocytes with lipopolysaccharide activates mitochondrial biogenesis and autophagy (Hickson-Bick et al., 2008), it is possible that the TNFα-induced changes in the mitochondrial network expressed as an increased proportion of tubular-shaped organelles also reflect a pro-survival mechanism (see Fig. 7). This assumption seems justified in view of the data indicating similar changes in gene expression patterns in endothelial cells exposed to LPS or TNFα. One is therefore tempted to assume that the response of neonatal rat cardiomyocytes to lipopolysaccharide may to some extent resemble their stimulation by TNFα (Magder et al., 2006). Contrary to the findings presented in this paper, TNFα applied at 4 ng/ml for 4 days was found to decrease the mitochondrial membrane potential in 3T3-L1 adipocytes. Despite that ROS production was elevated in those cells as it was in EA.hy926 cells studied here, thus the

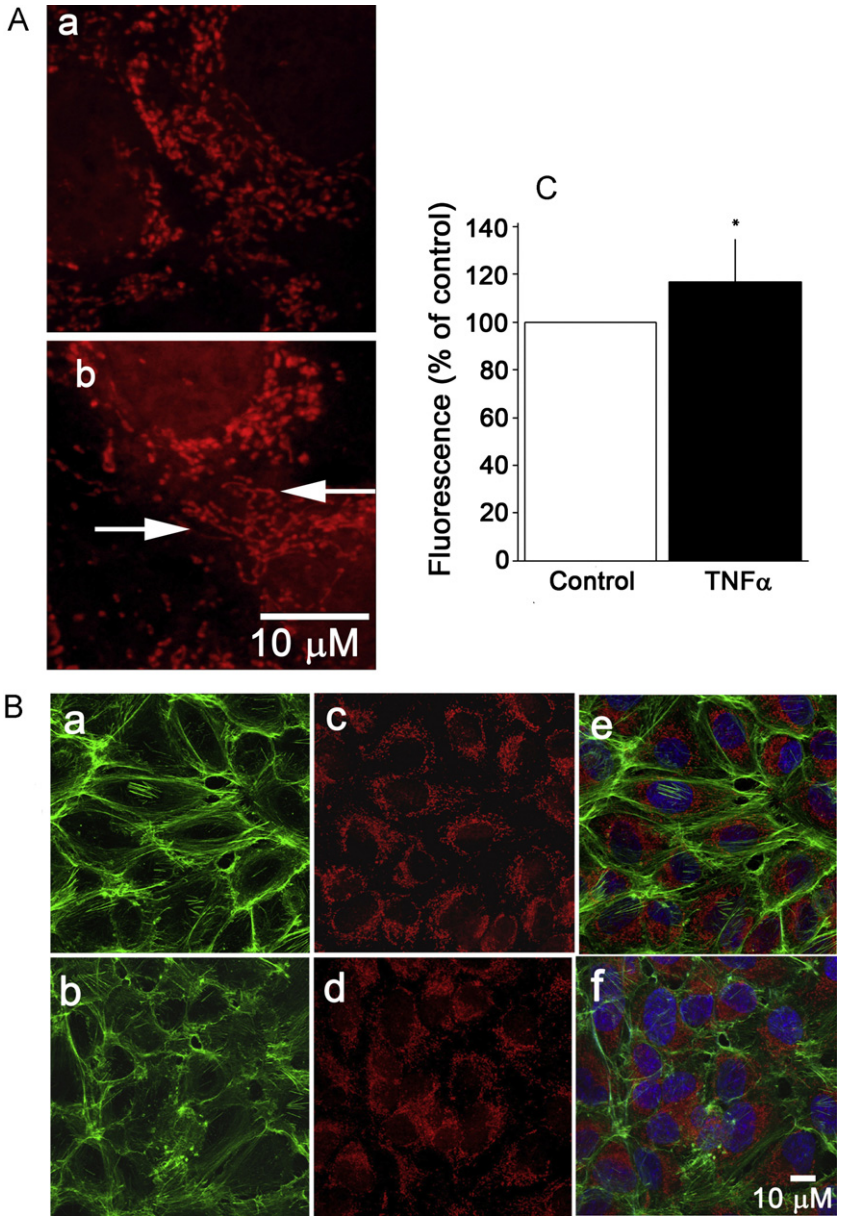

Fig. 7. Effect of TNFα on mitochondrial organization and total mitochondrial mass. (A) Mitochondrial organization. (a) Control; (b) TNFα-treated cells. Arrow indicates tubular mitochondria which appear upon TNFα treatment. (B) Actin cytoskeleton and mitochondria; (a and b) actin cytoskeleton in control and TNFα-treated cells, respectively; (c and d) mitochondria in control and TNFα-treated cells, respectively; (e and f) overlay. (C) Effect of TNFα on total mitochondrial mass estimated with MitoTracker Green. $n = 5$, $p < 0.04$.

mechanisms of ROS generation in EA.hy926 cells and 3T3-L1 adipocytes are likely to differ. Moreover, incubation of 3T3-L1 cells with TNFα caused a substantial reduction of PGC-1α level while the NRF-1 and TFAM-1 protein content was unchanged or slightly lower than in the control cells. Those changes suggested attenuated mitochondrial biogenesis in the TNFα-treated adipocytes (Chen et al., 2010). Again, tissue-specific response to TNFα is the most likely explanation of these different effects.

5. Conclusions

In conclusion, data presented in this paper indicate that pro-inflammatory stimulation of EA.hy926 endothelial cells with TNFα may induce pro-survival changes in mitochondrial organization and enhance the protein level of transcription factors necessary for mitochondrial protein synthesis accompanied with increased level of at least some respiratory chain proteins. These observations are in line with those concerning LPS-stimulated muscle cells and suggest a similar mechanism operating in endothelial cells.

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