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TNF-α mediates mitochondrial uncoupling and enhances ROS-dependent cell migration *via* NF-κB activation in liver cells

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ABSTRACT

Development of hepatocellular carcinoma (HCC) is accompanied by a continuous increase in reactive oxygen species (ROS) levels. To investigate the primary source of ROS in liver cells, we used tumor necrosis factor-alpha (TNF-α) as stimulus. Applying inhibitors against the respiratory chain complexes, we identified mitochondria as primary source of ROS production. TNF-α altered mitochondrial integrity by mimicking a mild uncoupling effect in liver cells, as indicated by a 40% reduction in membrane potential and ATP depletion (35%). TNF-α-induced ROS production activated NF-κB 3.5-fold and subsequently enhanced migration up to 12.7-fold. This study identifies complex I and complex III of the mitochondrial respiratory chain as point of release of ROS upon TNF-α stimulation of liver cells, which enhances cell migration by activating NF-κB signalling.

1. Introduction

Human hepatocellular carcinoma (HCC) is the 5th most common cancer occurring worldwide with prevalence in men and higher incidence rates in developing than developed countries [1]. Whereas about 80% of primary liver cancers develop through underlying Hepatitis B (HBV) and Hepatitis C (HCV) infections, other risk factors include smoking, diabetes, overweight and aflatoxin exposure in diet. Apart from known risk factors, several molecular mechanisms have been elucidated that contribute to HCC. This comprises alterations in signalling pathways, oncogene activation or decreased tumour suppressor gene expression.

Reactive oxygen species (ROS) such as superoxide anions are byproducts of respiration, generated by mitochondria. In recent years it became evident that ROS are not only byproducts of respiration but also can act as second messengers to activate signalling pathways and hence lead to alterations in gene expression [2–4]. Increased ROS levels are commonly described in enhanced liver disease and HCC [5–7]. However, the primary source of ROS generation in liver disease has not yet been fully elucidated. In addition, whether ROS act as second messenger in altering signalling pathways or expression of tumour suppressors, therefore reinforcing the development of HCC, remains to be elucidated.

HCC often develops through underlying infections accompanied by activation of the immune system. Increased expression of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) is associated with hepatocyte apoptosis but is also essential in hepatocyte proliferation [8] and liver regeneration [9]. By binding to its receptors (TNFR1/TNFR2), TNF-α activates two different signalling cascades either leading to apoptosis or proliferation [10]. In the latter case, TNF-α-stimulation leads to IKK activation resulting in IκBα phosphorylation and subsequent IκB degradation. NF-κB translocates into the nucleus inducing downstream gene targets including the pro-inflammatory chemokine IL-8. Activation of NF-κB ultimately results in enhanced proliferation and cell survival. In addition to its signalling modulatory effects, TNF-α is described to induce ROS production in mitochondria as well as to alter mitochondrial function by impairing membrane permeability [11,12].

Here, we investigated TNF-α-induced ROS production in liver cells and identified the respiratory chain as primary source of

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazine; HBV, hepatitis B virus; HCV, hepatitis C virus; H_2DCF-DA, dichlorofluorescein diacetate; NAC, *N*-acetylcysteine; ROS, reactive oxygen species; TMRE, tetramethylrhodamine ethyl ester; TNF-α, tumor necrosis factor-alpha

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ROS production. TNF-α-induced ROS production led to activation of NF-κB and enhanced cell migration of liver cells.

2. Materials and methods

Materials and methods are given in the Supplementary Data.

3. Results

3.1. Mitochondria are the main source of ROS production in liver cells

To identify the source of ROS production in liver cells, we established an *in vitro* cell system using the cytokine TNF-α to stimulate primary murine hepatocytes and two liver cancer cell lines (Hepa1–6 [murine], Huh-7 [human]). Different concentrations of TNF-α induced ROS in all cell types, however, 50 ng/ml TNF-α was most effective, inducing a 150% increase in ROS (Fig. 1A; Supplementary Fig. S1A). Of note, TNF-α did not induce cell death at concentrations used (data not shown). 50 ng/ml TNF-α was then applied throughout all experiments. Using the antioxidant *N*-acetylcysteine (NAC), TNF-α-induced ROS was dose-dependently inhibited in primary murine hepatocytes by 42.1% (±6.0; $P = 0.002$) and 57.8% (±4.4; $P < 0.001$), respectively (Fig. 1A) and also in Huh-7 cells (Supplementary Fig. S1A) and Hepa1–6 cells (data not shown). In addition, ROS was scavenged by the water soluble Vitamin E derivate Trolox (data not shown). To determine the primary source of the ROS signal, a variety of different inhibitors of the respiratory chain were applied to primary murine hepatocytes and liver cancer cells. The specific complex I inhibitor of the respiratory chain, Rotenone, dose-dependently blocked the TNF-α-induced ROS signal in hepatocytes by 71.8% (±18.4; $P = 0.007$) and 96.6% (±6.8; $P < 0.001$), respectively (Fig. 1B). Since primary hepatocytes were not transfectable in culture, this result was validated in Hepa1–6 and Huh-7 cells using specific siRNAs against NDUFAF1, an essential assembly factor for complex I [13]. NDUFAF1 siRNA transfection also inhibited the TNF-α-induced ROS signal by over 95% (±12.1; $P < 0.001$; Fig. 1C; Supplementary Fig. S1B and C). In addition, ROS production was also dose-dependently inhibited by the complex III inhibitor Antimycin A (Fig. 1B; Supplementary Fig. S1B). Here, ROS production generated by TNF-α-stimulation was reduced to 36.1% (±20.6) and 17.7% (±11.9; $P = 0.02$), respectively (Fig. 1B). Using Antimycin A at a concentration of 1 μg/ml resulted in slight toxicity, hence 0.5 μg/ml was used for subsequent experiments. Using a pool of siRNAs against the complex III assembly factor BCS1L [14], this also reduced TNF-α-induced ROS generation in Hepa1–6 cells (54.3%, ±4.1; $P < 0.001$) and Huh-7 cells (Fig. 1C; Supplementary Fig. S1C). To further demonstrate the important role of complex I and complex III in TNF-α-mediated ROS production in liver cells, we used inhibitors against complex II, complex IV and the F_0F_1-ATPase. As expected, the complex II inhibitor Atpenin A5 significantly reduced TNF-α-induced ROS production. Of note, from complex II additional electrons are delivered to complex III *via* the quinone pool. In contrast Azide (complex IV inhibitor) and Oligomycin (ATPase inhibitor) did not show any effect (Supplementary Fig. S1D). Reducing TNF-α-induced ROS production using specific chemical inhibitors and siRNA against components of the respiratory chain identifies the mitochondrial respiratory chain, involving complex I and III, as primary source of TNF-α-specific ROS production in liver cells.

3.2. TNF-α mimics a mild uncoupling effect on mitochondria in liver cells

Mitochondria were identified as source of TNF-α-induced ROS production. Therefore, we further investigated the influence of TNF-α on mitochondria. TNF-α-treatment led to a significant reduction in membrane potential in murine hepatocytes (63% ±3.5; $P < 0.001$; Fig. 2A). In addition, TNF-α treatment reduced ATP production in murine hepatocytes by 34.8% (±2.8; $P = 0.006$) (Fig. 2B). Reduction in membrane potential as well as ATP depletion are described phenomena in mitochondrial uncoupling [15]. To further investigate alterations in mitochondrial physiology in hepatocytes we studied the activities of respiratory chain complexes, electron flux and mitochondrial respiration. Whereas no differences were seen after TNF-α treatment in the activity of complex I, II, III and IV and in electron flux from complex I to complex III (data not shown), an increased electron flux of 19.7% (±28.7; $P = 0.03$) from complex II to complex III was observed upon TNF-α-treatment in murine hepatocytes (Fig. 2C). Also, mitochondrial oxygen consumption was increased after TNF-α treatment (173.4% ±28.7) compared to control-treated cells (60.5 ± 8.6; $P = 0.02$; Fig. 2D). Here, we show an alteration of mitochondrial integrity by TNF-α-treatment in liver cells that mimics mitochondrial uncoupling events as seen with the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) (Fig. 2A and B). Therefore, we identify that TNF-α treatment leads to a mild uncoupling of mitochondrial respiration in liver cells.

3.3. TNF-α-induced ROS production alters cell migration in liver cells

The previous experiments showed that TNF-α induces ROS production and alters mitochondrial function. Therefore, we investigated how TNF-α-mediated ROS production physiologically affects liver cells. Due to the non-proliferative capacity of primary hepatocytes in cell culture, we performed wound healing assays in Hepa1–6 cells after TNF-α-treatment with or without Rotenone and Antimycin A. Migratory potential was then evaluated as a measure of closure of the gap within a 16 h time frame (Fig. 3A). TNF-α increased migration in Hepa1–6 cells compared to control-treated cells (4.6-fold ±3.0) and treatment with Rotenone inhibited this effect (4.2-fold decrease ±0.1) (Fig. 3A). Similar results were obtained using Antimycin A (Fig. 3B). Here, TNF-α increased migration in Hepa1–6 cells by 12.7-fold (±3.8; $P = 0.04$) and additional treatment with Antimycin A almost completely reversed this effect (14.8-fold decrease ±0.1; $P = 0.04$) (Fig. 3B). To ensure that the effects seen with Rotenone and Antimycin A are not due to cytotoxicity, the ROS scavenger NAC and Trolox were used as control. Also, with NAC (Fig. 3; Supplementary Fig. S2) and Trolox (data not shown), migration was inhibited (19.6-fold decrease ±0.3; $P = 0.04$). Thus, complex I and complex III activity plays a crucial role in controlling the proliferative capacity of liver cells.

3.4. NF-κB signalling is affected by TNF-α-induced mitochondrial ROS production

TNF-α is known to activate the ROS-dependent transcription factor NF-κB [2], which in turn leads to enhanced cell growth and proliferation. We therefore investigated the expression of NF-κB-target genes after TNF-α-treatment. The NF-κB-dependent target genes *Cxcl1*, *IκBa* and *A20* were upregulated upon TNF-α treatment in murine hepatocytes (Fig. 4A and C). This increase in mRNA levels could be reversed by either pre-treating cells with NAC (Supplementary Fig. S3A) or Rotenone (Fig. 4A) and Antimycin A (Fig. 4C), respectively. In addition, TNF-α treatment also increased the expression of IL-8 and *IκBa* in Huh-7 cells and this was also reversed by both Rotenone and Antimycin A (Supplementary Fig. S3B). In HCC, the NF-κB target gene IL-8 is involved in regulation of the expression of genes involved in invasion and metastasis [16]. To also associate our findings with a clinical setting, we investigated human tissue samples. Comparing expression data of 67 human HCC with 10 normal control liver tissues [17] (GEO database, accession GSE50579), a significant (*t*-test,

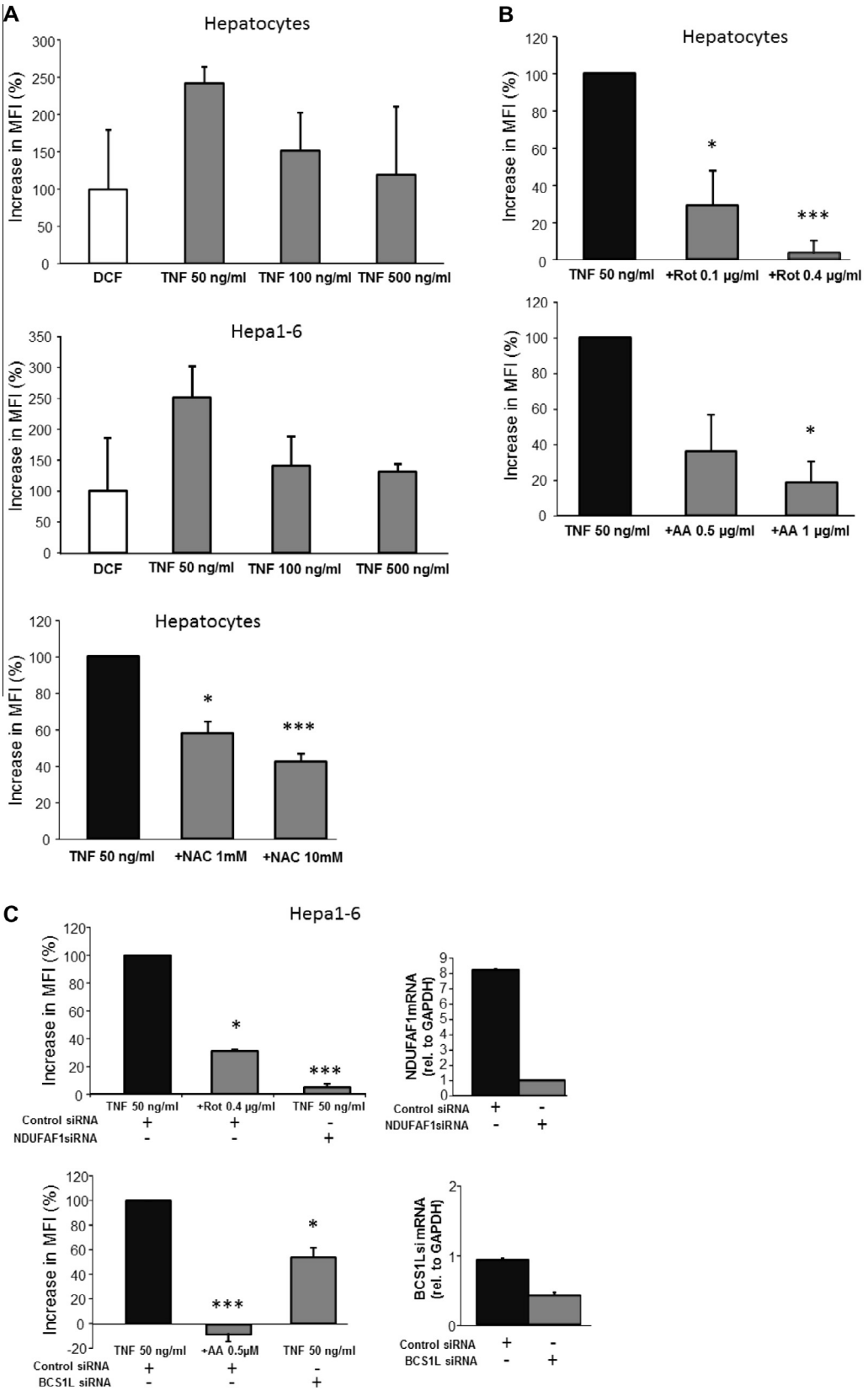

Fig. 1. Mitochondria are the main source of TNF-α-induced ROS production in liver cells. (A) ROS generation after TNF-α-stimulation was measured in primary murine hepatocytes and Hepa1–6 cells using H_2DCF-DA after administration of different concentrations of TNF-α for 1 h. In addition, primary murine hepatocytes were pre-treated with NAC for 30 min and subsequently treated with TNF-α for 1 h. H_2DCF-DA-only or NAC-only treated cells served as control and % decrease is presented relative to TNF-α-only treated cells. (B) The source of TNF-α-mediated ROS production was investigated in primary hepatocytes using Rotenone (Rot) and Antimycin A (AA). Primary murine hepatocytes were pre-treated with different Rotenone and Antimycin A concentrations for 30 min, respectively, and subsequently treated with TNF-α for 1 h. H_2DCF-DA-only or Rotenone-only or Antimycin A-only treated cells served as control and % decrease is presented relative to TNF-α-only treated cells. (C) Hepa1–6 cells were transfected with siRNAs against NDUFAF1 and BCS1L and TNF-α-induced ROS production was measured. H_2DCF-DA-only stained cells served as control. ROS measurements were performed as in (B). All experiments were performed in triplicate with three technical replicates per sample. $^{*}P < 0.05$; $^{***}P < 0.001$.

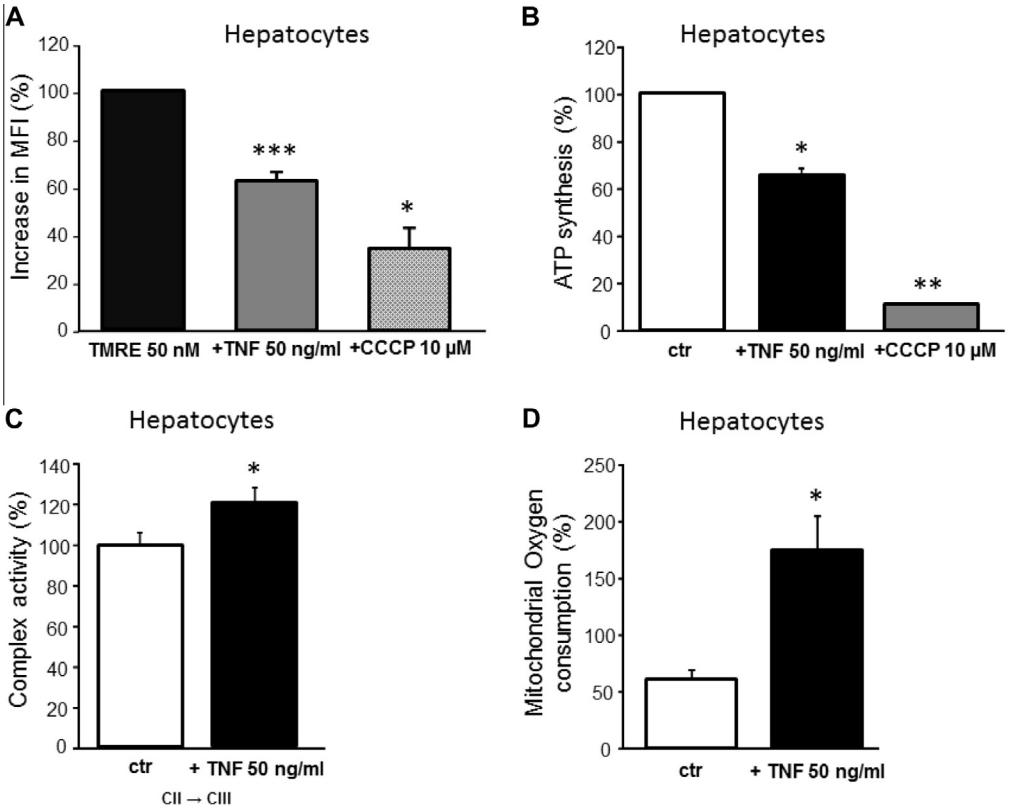

Fig. 2. TNF-α mimics an uncoupling effect on mitochondria in liver cells. (A) Mitochondrial membrane integrity was measured using TMRE. Mitochondrial depolarisation is presented as MFI and alterations in fluorescence were measured after administration of TNF-α for 1 h. TMRE-only stained cells were used as control. CCCP was used as positive control. (B) Intracellular ATP content in primary murine hepatocytes in TNF-α-treated cells (50 ng/ml, 1 h) compared to control-treated cells. Results are shown as % increase relative to control, where control cells were set to 100%. CCCP was used as positive control. (C) Electron flux from complex II (CII) to complex III (CIII) was measured in primary murine hepatocytes after 1 h TNF-α treatment (50 ng/ml). Results are shown as complex activity in % relative to control-treated cells. (D) Mitochondrial oxygen consumption was measured after 1 h TNF-α treatment (50 ng/ml) compared to control-treated cells. Results are shown as mitochondrial oxygen consumption in %, where mitochondrial oxygen consumption was calculated by subtracting the "NaN_3-insensitive oxygen consumption rate" from the "total oxygen consumption rate". All experiments were performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

$P = 0.0441$) increase of IL-8 gene expression (2.938-fold induction) was observed. To further validate a TNF-α – dependent activation of NF-κB due to ROS production, we used a NF-κB luciferase reporter system. Here, TNF-α activated NF-κB, which was almost completely inhibited by pre-treating the cells with specific ROS scavengers or siRNAs against important assembly factors for complexes of the respiratory chain (Supplementary Fig. S3C). This demonstrates a complex I- and/or complex III-dependent activation of the NF-κB pathway upon stimulation with TNF-α in liver cells.

We also investigated NF-κB-activation on protein level. TNF-α-stimulation increased IκBα and P65 phosphorylation after 10 min (Fig. 4B and D). Both, Rotenone and Antimycin A reversed this activation, further validating our findings of mitochondrial alterations and ROS-dependent NF-κB-activation upon TNF-α-treatment (Fig. 4B and D).

3.5. TNF-α-induced ROS production enhances cellular migration via NFκB-activation

We reasoned that increased cell migration was due to the activation of NF-κB. To test this hypothesis, Hepa1–6 cells were pre-treated with an NF-κB-activation inhibitor, followed by TNF-α-stimulation and the migration potential was assessed by wound healing. Application of an NF-κB-activation inhibitor reversed TNF-α-induced migration (6.5-fold ±2.8) in Hepa1–6 cells by 6.5-fold (±0.04) (Fig. 5A) to a similar extent compared to treatment with the anti-oxidant NAC (Supplementary Fig. S2).

4. Discussion

In the present study, we show that TNF-α-induced ROS in liver cells is mainly generated by mitochondria and that ROS release is exerted by complex I and/or complex III of the respiratory chain. TNF-α stimulation altered mitochondrial integrity by decreasing mitochondrial membrane potential and ATP synthesis, by increasing electron flux from complex II to complex III as well as by enhancing mitochondrial respiration. In addition, we showed that TNF-α-induced ROS production leads to NF-κB-activation and hence activation of pro-survival signals which increased cellular migration of liver cells.

TNF-α is a pro-inflammatory cytokine that was first described as product of cytotoxic activity of lymphocytes [18]. TNF-α, and other members of the TNF-superfamily are secreted by T and B cells, monocytes and macrophages. By binding to the receptors TNFR1/TNFR2, TNF-α can either activate pro-apoptotic or anti-apoptotic signalling [19]. Cytokines, including TNF-α, are released due to response to a variety of different cellular events such as inflammation and carcinogen-induced injury [20]. As an immune cell-enriched organ, the liver is prone to inflammation and it is established that chronic infections with HBV and HCV are major risk factors for the development of HCC [21]. In line with this, increased TNF-α expression as well as elevated levels of TNFR1/TNFR2 are described to be elevated in HCC patients [22–24]. Apart from its pro-inflammatory actions, there is also evidence that TNF-α production increases oxidative stress in a variety of different

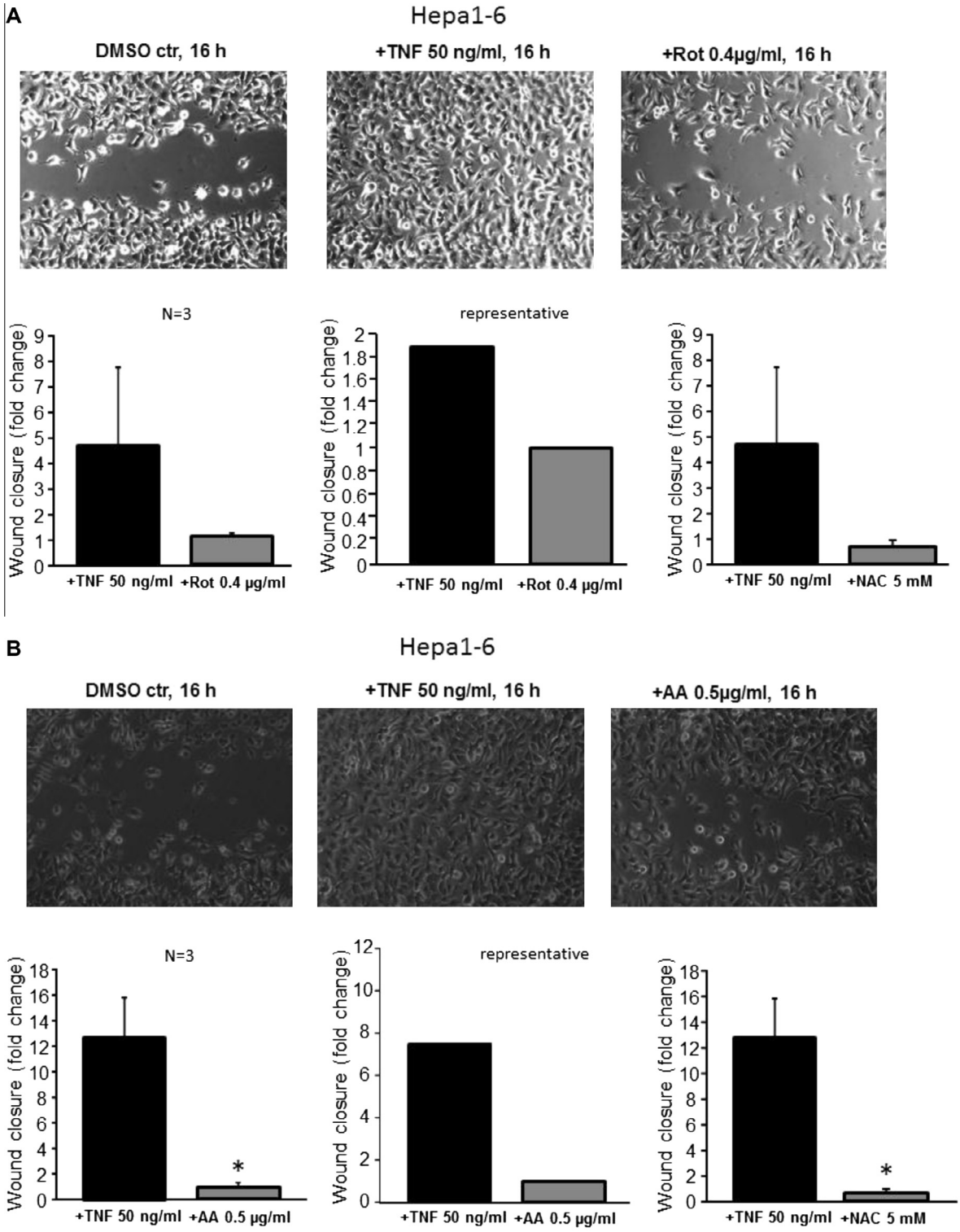

Fig. 3. TNF-α-induced ROS production alters cell migration in liver cells. Cellular migration was measured using wound healing assays. Hepa1–6 cells were either pre-treated with Rotenone (A) or Antimycin A (B), followed by 16 h TNF-α stimulation. (A, upper panel) Representative image. (A, lower panel) Mean quantification of Hepa1–6 cells treated with Rotenone and TNF-α ($n = 3$). (A, lower panel) Quantification of one representative experiment of Hepa1–6 cells treated with Rotenone and TNF-α. (A, lower panel) Mean quantification of Hepa1–6 cells treated with NAC and TNF-α ($n = 3$). (B, upper panel) Representative image. (B, lower panel) Mean quantification of Hepa1–6 cells treated with Antimycin A and TNF-α ($n = 3$). (B, lower panel) Quantification of one representative experiment of Hepa1–6 cells treated with Antimycin A and TNF-α. (B, lower panel) Mean quantification of Hepa1–6 cells treated with NAC and TNF-α ($n = 3$). The open area of the gap was recorded at time point 0 and after 16 h TNF-α treatment. Migration was expressed as fold change of wound closure where differences in wound closure were calculated relative to control (DMSO-treated cells). Experiments were performed in triplicate with at least three technical replicates per sample. $^{*}P < 0.05$.

cells [25,26]. We could induce the production of ROS in primary murine hepatocytes and liver cancer cells already after short-term treatment with TNF-α. However, in contrast to many reports, TNF-α-produced ROS did not induce cell death, which resembles the

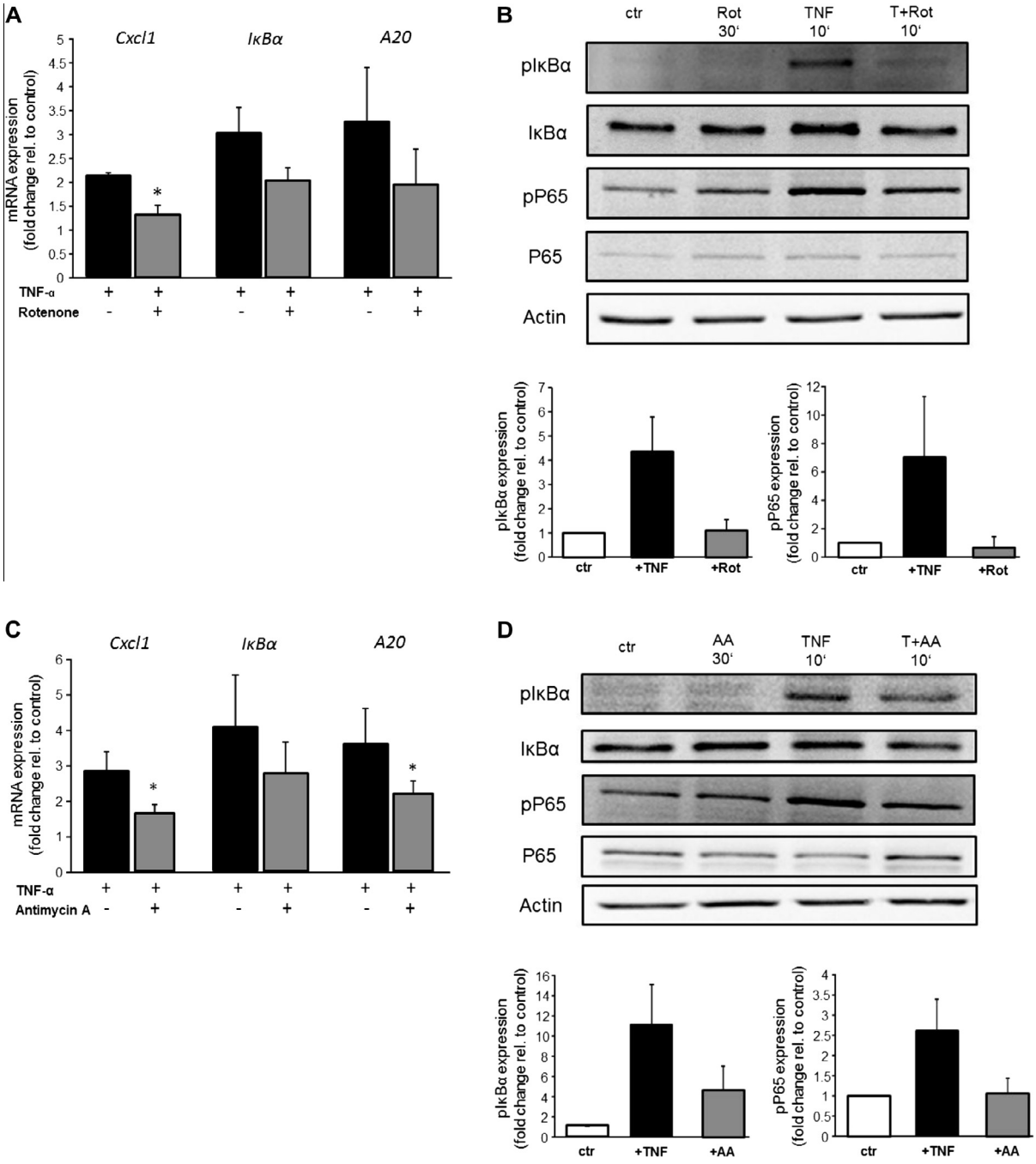

Fig. 4. NF-κB signalling is affected by TNF-α-induced mitochondrial ROS production. *Cxcl1*, *IκBα* and *A20* mRNA expression was measured by qRT-PCR in TNF-α-treated cells with or without pre-treatment with Rotenone (A) or Antimycin A (C), respectively. *GAPDH* was used for normalisation. The results are expressed as fold induction (±S.E.M.) relative to control. DMSO-treated cells, Rotenone-only or Antimycin A-only treated cells served as control (=1). All experiments were repeated in duplicate with cDNA synthesized from three independent RNA extractions. pIκBα, total IκBα, pP65 and total P65 were measured in TNF-α-treated cells with or without pre-treatment with Rotenone (B) or Antimycin A (D) for indicated time points. (Upper panel) Representative Western blots are shown. (Lower panel) Protein expression was calculated relative to total IκBα and total P65 expression, respectively. Results are shown as fold change (±S.E.M.). DMSO-treated cells, Rotenone-only or Antimycin A-only treated cells served as control (=1). All experiments were repeated in triplicate. $^{*}P < 0.05$; $^{***}P < 0.001$.

pleiotropic effects of this cytokine [27–29] and suggests a role of ROS as second messenger. To elucidate the primary source of TNF-α-induced ROS production in liver cells, we applied inhibitors against different complexes of the respiratory chain and identified mitochondria as primary source of ROS production with complex I and/or complex III as key points of ROS generation. Several studies identified mitochondria as main source of ROS production in different cellular processes. Whereas complex I was identified as primary point of release in activation-induced ROS generation in human T cells [3], complex III-dependent ROS generation was essential to initiate adipocyte differentiation [30]. We found complex I and/or complex III as points of ROS release in our experimental system, which indicates a cell type-dependent and stimulus-specific involvement of different complexes of the respiratory chain in ROS-dependent signalling. However, mitochondrial ROS sources can crosstalk to other ROS sources such as NADPH oxidases [3]. Hence other ROS sources may also be activated upon TNF- α in liver cells.

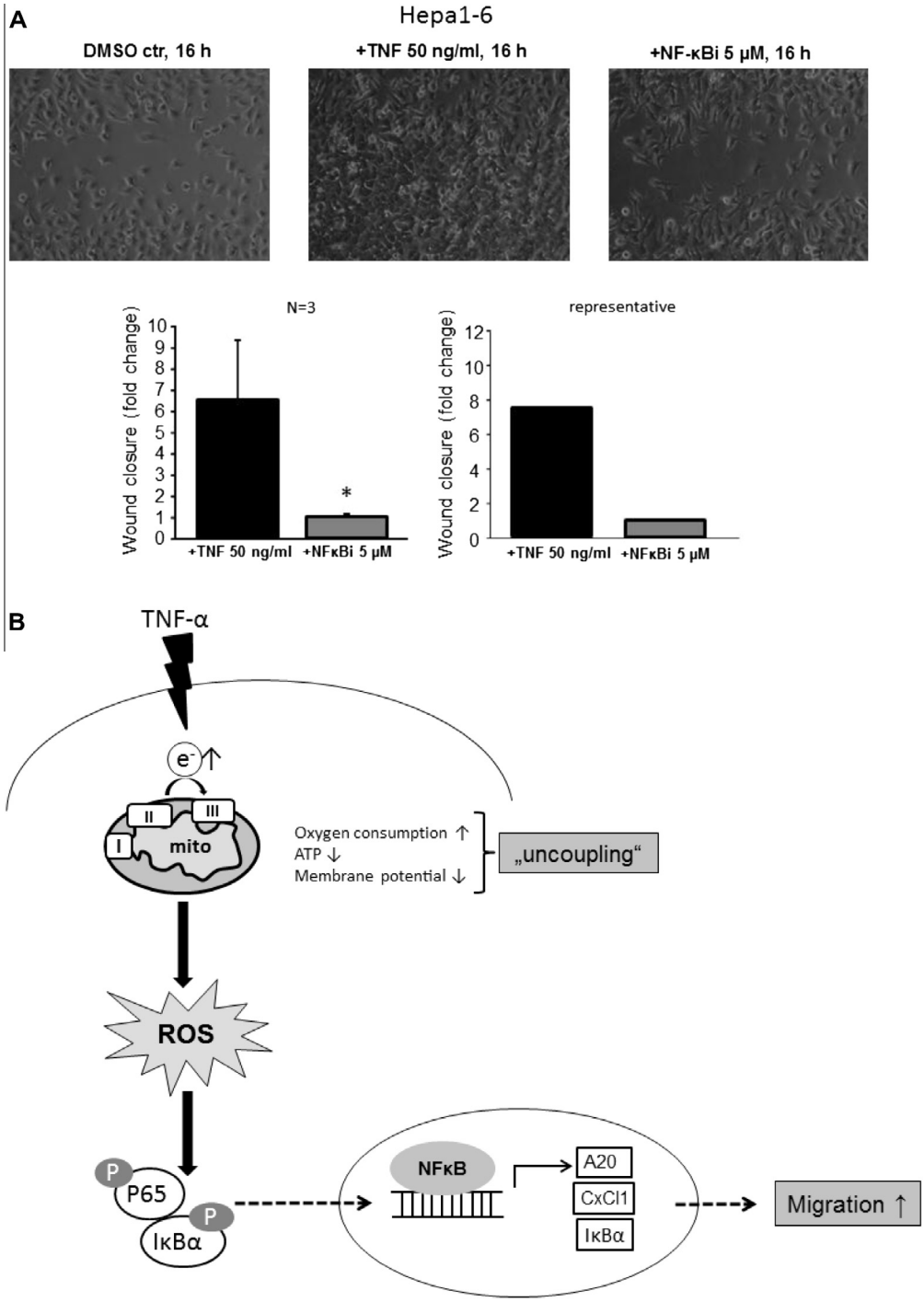

Fig. 5. TNF-α-induced ROS production enhances cellular migration *via* NF-κB-activation. Cells were pre-treated with NF-κB-activation inhibitor (5 μM), followed by 16 h TNF-α stimulation. (A, upper panel) Representative image. (A, lower panel) Mean quantification of Hepa1–6 cells treated with NF-κB-activation inhibitor and TNF-α ($n = 3$). (A, lower panel) Quantification of one representative experiment of Hepa1–6 cells treated with NFκB-activation inhibitor and TNF-α. The open area of the gap was recorded at time point 0 and after 16 h TNF-α treatment. Migration was expressed as fold change of wound closure where differences in wound closure were calculated relative to control (DMSO-treated cells). Experiments were performed in triplicate with at least three technical replicates per sample. $^{*}P < 0.05$. (B) Schematic representation of the proposed mechanism of ROS- and NF-κB-dependent migration of liver cells.

In primary murine hepatocytes, TNF-α directly affected mitochondria by decreasing membrane potential and ATP synthesis, by accelerating electron flux from complex II to complex III and by enhancing mitochondrial respiration (Fig. 5B). Particularly, a breakdown of the mitochondrial membrane potential and ATP depletion are per definition indicators of mitochondrial uncoupling, which is described for uncouplers of oxidative phosphorylation, such as CCCP [15,31,32]. In addition, many studies also observed enhanced electron flux and increased mitochondrial respiration upon uncoupling [33–35]. Therefore, we identify TNF-α as a mild uncoupler in liver cells. In line with this, TNF-α was described to alter mitochondrial integrity and mitochondrial electron transfer was completely diminished by TNF-α in mouse fibroblasts [36]. Also, mitochondrial ultrastructure was degenerated [12] and uncoupling respiration of

liver mitochondria was shown prior to the cytotoxic effects of TNF-α [11]. Hereby, TNF-α may activate different receptor-dependent pathways for example *via* RIP [37] or other so far not identified molecules.

Pro-survival signals mediated by TNF-α may indicate a role of NF-κB-activation [19]. NF-κB-activation is a described phenomenon in a variety of different cancers, including cancers of lymphoid tissues and solid tumours such as HCC [38]. In the present study, we showed that TNF-α activates NF-κB in a ROS-dependent manner, since both the complex I inhibitor Rotenone and the complex III inhibitor Antimycin A inhibited NF-κB-target gene expression and phosphorylation of IκBα and P65. Oxidative stress activates NF-κB in different cell types [39,40], however, TNF-α-induced ROS release through complex I and complex III of the mitochondrial respiratory chain leading to NF-κB-activation in liver cells has not been previously shown. In addition, enhanced cellular migration in liver cells was dependent on the activation of NF-κB, as shown by the use of a specific NF-κB-activation inhibitor. NF-κB-dependent cell migration was reported by several groups [41], highlighting the key role of NF-κB activation in the development of cancer, including HCC.

Increased ROS production is observed at different stages of liver disease and in HCC. In addition, enhanced oxidative stress due to TNF-α expression in Kupffer cells bears a major clinical problem in the course of hepatic ischemia/reperfusion injury [42]. Here, we show that TNF-α-induced mitochondrial ROS is released by complex I and/or complex III of the mitochondrial respiratory chain in liver cells. Furthermore, TNF-α-induced ROS production enhances cellular migration through the activation of NF-κB signalling elucidating a major ROS-dependent signalling cascade initiated by the pro-inflammatory cytokine TNF-α in liver cells (Fig. 5B). Our findings suggest that TNF-α-induced ROS signalling in liver cells may constitute a therapeutic target occurring during inflammation, hepatic ischemia/reperfusion injury and cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.11.033.

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