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Letter to the Editor

Loss of apoptosis-inducing factor leads to an increase in reactive oxygen species, and an impairment of respiration that can be reversed by antioxidants

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Dear Editor,

Since its discovery, the role of apoptosis-inducing factor (AIF) in the apoptotic process has been studied exhaustively.[1–3] At present, it is recognized that after a proapoptotic stimulus, AIF translocates from the mitochondrial intermembrane space to the cytosol and thence to the nucleus where it induces peripheral chromatin condensation and high molecular weight (50 kbp) DNA fragmentation.[3,4] AIF, itself, has no intrinsic nuclease activity, and how AIF causes these changes remains unclear. Extra-mitochondrial targeting of AIF, as well as microinjection of recombinant AIF protein into cells, or addition of AIF to isolated nuclei, generally leads to the generation of apoptotic phenotypes such as chromatin condensation, mitochondrial membrane potential dissipation and phosphatidylserine exposure on the cell surface.[4,5] The crystal structure of human AIF reveals two important regions, the first region exhibits an oxidoreductase enzyme activity and the second region represents a putative DNA-binding site.[6] The oxidoreductase catalytic region is structurally homologous to diphenyl dioxygenase (bphA4), a bacterial oxygenase-coupled NAD-dependent ferredoxin reductase that manifests a similar fold to the eucaryotic glutathione reductase family of enzymes.[7] In functional assays, AIF acts as an NADH oxidase, catalyzing the net transfer of electrons from NADH to O_2 and generating superoxide.[8] Because it can stably bind FAD, AIF falls into the category of flavoproteins. Importantly, the oxidoreductase-active region of AIF is not essential for its apoptogenic activity.[8] It was recently reported that the *Harlequin* (Hq) mutant mouse, which displays progressive degeneration of cerebellar and retinal neurons with aging, harbors a proviral insertion in the first intron of the *AIF* gene, provoking an 80% decrease in AIF expression.[9] Interestingly, cerebellar granule cells from the Hq mutant mice were more susceptible to peroxide-induced apoptosis than wild-type counterparts, suggesting that AIF serves as a free radical scavenger (and not a superoxide producer), and could therefore ameliorate neuronal apoptosis induced by hydrogen peroxide.[9] This finding suggests that AIF could play a supportive role in respiration, as the electron transport chain (ETC) is the main source of reactive oxygen species (ROS). It follows that mutations in AIF might produce a defect in mitochondrial respiration, through increased production of ROS.[10,11] It has been shown that AIF can catalyze the reduction of cytochrome *c* in the presence of NADH *in vitro*, implying that cytochrome *c* is a possible electron acceptor for AIF.[8] Moreover, a proteomic study identifies the presence of AIF in a purified fraction of the complex IV of the mitochondrial oxidative phosphorylation system.[12] A very recent study published during the course of this work proposed that AIF is involved in the biogenesis and/or maintenance of the ETC, as AIF mutations resulted in a loss of Complex I and Complex III polyproteins.[13] Of special significance in this study was the result that loss of AIF compromised oxidative phosphorylation. Here we examined in cells whether depletion of AIF would lead to increases in free radical formation. We observed, for the first time, that there was a direct association between the increases in ROS, after AIF depletion, and the reduction in O_2 consumption.

To investigate the possible relationship between AIF and ROS, we used vector-driven small interfering RNA (siRNA)[14] to reduce the expression of the AIF transcript. Two unique AIF-specific siRNAs (AIF-1, GTACTGATTGTATCTGAA GAT, and AIF-2, GTAGTACAGCTGGATGTGAGA), but not control siRNA, effectively reduced the steady-state levels of AIF protein in both Hep3B cells (Figure 1a(i, top)) and HeLa cells (Figure 1a (ii, top)), and this was paralleled by an approximate two-fold increase in cellular H_2O_2 levels (Figure 1a (i and ii, bottom)). As AIF is a mitochondrial protein, we thought it reasonable to speculate that these observed increases in ROS were originating from the ETC. To test this claim, we first generated Hep3B cells that had been depleted of mitochondrial DNA (termed ρ^0) by long-term culture in the presence of ethidium bromide.[15] After testing for loss of respiratory function (results not shown), we repeated siRNA transfection in both Hep3B ρ^+ and ρ^0 cells. As shown in Figure 1a (iii), siRNA transfection in both ρ^+ and ρ^0 cells resulted in a robust knockdown of AIF protein levels. However, the evident increase in H_2O_2 levels observed in the ρ^+ cells was not apparent in ρ^0 cells, pointing to the ETC as the source of ROS in AIF-depleted cells. In order to broaden these findings in a more controlled manner, we generated stable Hep3B cell lines containing integrated plasmids expressing siRNAs against AIF. From a range of stable cell lines, we chose AIF-1-10 and AIF-2-4 for further study. These cell lines demonstrated a consistent and robust silencing of AIF in long-term culture (Figure 1b). As controls for our continuing experiments, we used also cell lines stably

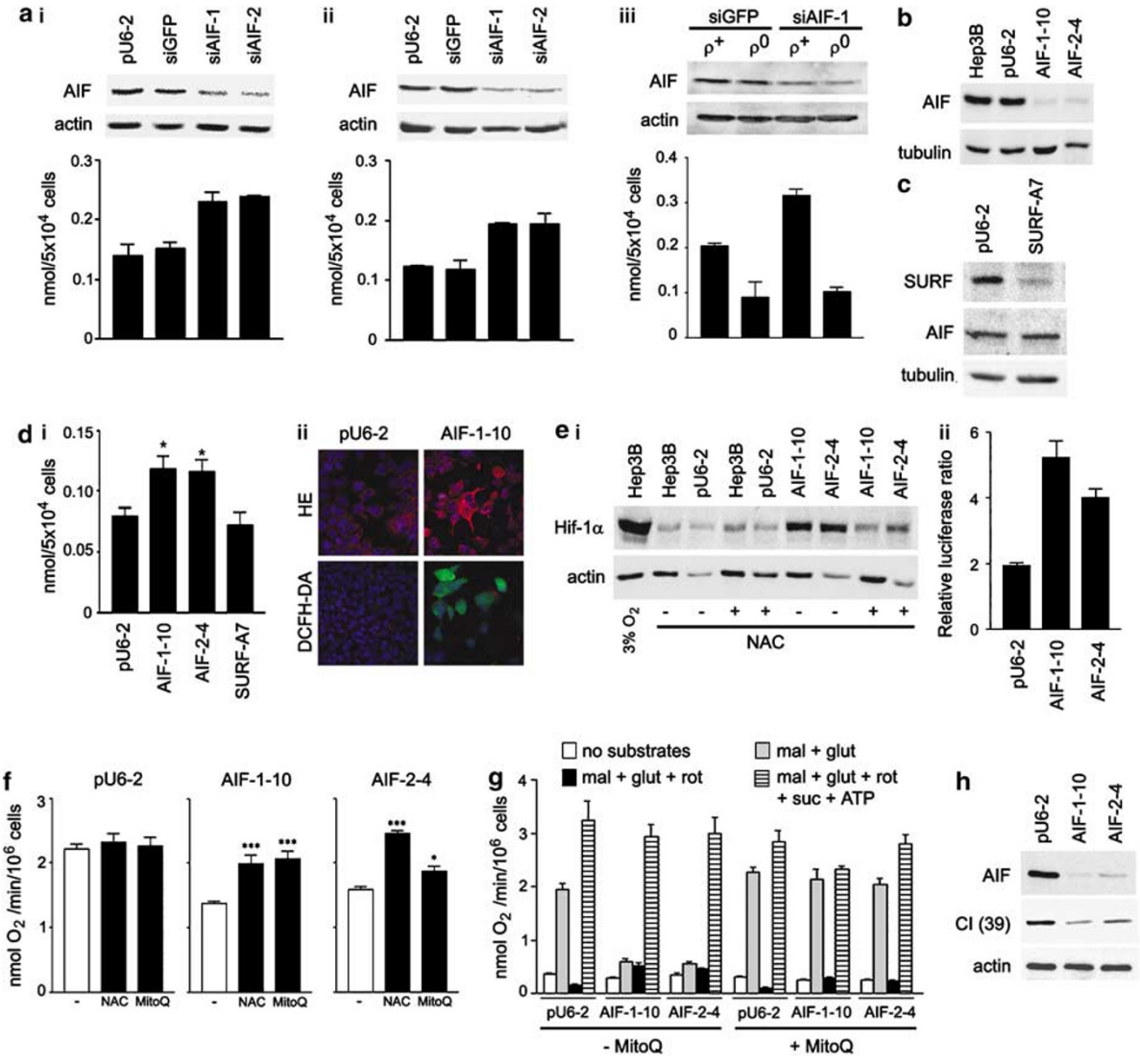

Figure 1 Antioxidant therapy rescues the respiration defect in AIF-silenced cells. (**a**) Hep3B (i), HeLa (ii) or Hep3B-ρ^0 (iii) cells, grown under standard conditions, were transfected twice at 24 h intervals with one of two siRNAs that specifically target the AIF gene (10 μg vector per T-25cm^2 flask, at 60% confluence, using Lipofectamine™ 2000, Invitrogen, Carlsbad, CA, USA). Control transfections included an siRNA targeted to GFP,[25] and also empty vector. At 48 h after the second transfection, cells from one half of the flask were harvested for Western blot analysis of total protein extracts using anti-AIF (ProSci. Inc., Poway, CA, USA) and anti-actin (Sigma, St. Louis, MI, USA) antibodies, and the remaining one half of the flask was harvested for H_2O_2 measurement (Amplex Red Hydrogen Peroxide/Peroxidase assay, Molecular Probes, Eugene, OR, USA). H_2O_2 measurements (mean $\pm$ S.E.M.) are typical of three experiments. (**b**) AIF silencing is sustained in stable cell lines. Western blot analysis of stable lines AIF-1-10, AIF-2-4 and pU6-2 using anti-AIF and anti-tubulin (Sigma) antibodies. Also shown is the Hep3B cell line for comparison. Cells were cultured for 8 weeks before preparation of total cellular protein extracts. (**c**) Western blot analysis using anti-AIF, anti-tubulin and anti-SURF1 (Molecular Probes) antibodies, of control cell line SURF-A7, showing that SURF1 protein is silenced but AIF protein is at normal levels. (**d**) ROS measurement in stable cells. (i) H_2O_2 was measured as described above, reported as the mean $\pm$ S.E.M. ($n = 6$, $^*P < 0.05$). (ii) Confocal fluorescence microscopy of cell lines pU6-2 and AIF-1-10 is shown. Cells were stained with DCFA-DA and HE for 30 min at 37°C protected from the light. Cellular fluorescence intensity was visualized using a Radiance 2100 Confocal microscope (Bio-Rad, Hampstead, UK), and digital image analysis was carried out with Laserpix software (Bio-Rad). The mean fluorescence intensity was captured per image (404 μm^2) corresponding to 25–35 cells. At least three images were captured per experiment. Results (mean $\pm$ S.E.M.) from three experiments were expressed as the % change in fluorescence to control. (**e**) HIF-1α is stabilized and active in siAIF cell lines. (i) Western blot analysis, using an anti-HIF-1α antibody (BD Transduction Laboratories , Lexington, MI, USA) in cell lines cultured with or without the addition of 5 mM NAC. As a positive control for HIF-1α stabilization, we included protein extracted from hypoxia-treated Hep3B cells. (ii) Cells (1×10^5) were transiently transfected with 200 ng of pGK-HRE1 and 75 ng of pHTK-RL (Promega Corp., Madison, WI, USA) in 24-well culture dishes. After 24 h, cell luminescence was measured using the STOP and GLOW™ reporter assay kit (Promega). Results were corrected for the activity of *Renilla* luciferase and represent the mean $\pm$ S.E.M. of three separate experiments. (**f**) Respiration in AIF-silenced cells can be rescued with antioxidants. Cells were cultured or not, for 16 h with 5 mM NAC or 0.5 μM MitoQ before oxygen consumption measurements. Oxygen consumption rates of 2–3 million cells, suspended in HBSS, were measured with a calibrated Clark-type electrode ($^{***}P < 0.001$, $^*P < 0.05$). (**g**) Oxygen consumption rates in digitonin-permeabilized cells. Cells were permeabilized with 100 μg/ml digitonin for 2 min and transferred to the Clark-type electrode. After recording basal oxygen consumption rates for 2 min, electron donor substrates and inhibitors were added sequentially. Results represent mean $\pm$ S.E.M. of 3–5 determinations. (**h**) Reduced expression of a Complex I subunit in siAIF cell lines. Western blot analysis using anti-AIF, anti-actin and anti-39 kDa (Molecular Probes Cat. A21344)

integrated with either empty vector only (pU6-2) or a vector-based siRNA toward the SURF1 gene (SURF-A7; Figure 1c). We examined whether the increased levels of ROS, upon AIF silencing, were also apparent after long-term culture of cells, as continuous exposure to elevated ROS can lead to upregulation of antioxidant defenses.[16] We measured ROS levels in AIF-1-10 and AIF-2-4 and compared these to the control pU6-2 and SURF-A7 cell lines. The results shown in Figure 1d (i) demonstrated that ROS levels were increased in the stable cell lines compared to controls ($P<0.05$). To complement this result, we analyzed AIF-1-10 and pU6-2 cells by confocal microscopy[17] using two redox-sensitive fluorescent probes. The results shown in Figure 1d (ii) reveal that both superoxide, measured by hydroethidium (HE, 10 μM), and peroxides, measured by 2′,7′-dichlorodihydrofluorescein (DCFH-DA, 0.4 μM), were significantly increased in the AIF-1-10 cells compared to control (194 $\pm$ 36%, HE; 271 $\pm$ 53%, DCFH-DA). Collectively, these results strongly suggested to us that an outcome of AIF knockdown is the increased production of mitochondrial ROS.

To support our findings so far, we next examined the consequences of AIF knockdown for the activity of HIF-1α, a ROS-sensitive transcription factor.[18] Given the evidence that HIF-1α protein stabilization can be controlled by mitochondrial ROS levels,[18] we examined the steady-state levels of HIF-1α protein in AIF-silenced cell lines. We cultured cells for 3 h in the presence or absence of the broad-range antioxidant, *N*-acetyl cysteine (NAC, 5 mM) and performed Western blot analysis on whole-cell extracts using an antibody directed to HIF-1α. The results shown in Figure 1e (i) reveal that whereas the cell lines Hep3B and pU6-2 expressed only basal levels of HIF-1α, both AIF-1-10 and AIF-2-4 expressed increased steady-state levels of HIF-1α, which could be reduced by prior incubation with NAC. Semiquantitative RT-PCR analysis showed that HIF-1α mRNA was unchanged in the AIF knockdown cells (results not shown). Extending these findings, we used an HIF-1α reporter construct[19] and show that HIF-1α protein in cell lines AIF-1-10 and AIF-2-4 was transcriptionally active, with reporter levels $>$2-fold higher in both AIF-silenced cell lines compared to the pU6-2 control (Figure 1e (ii)). Overall, these data suggested that the increased levels of HIF-1α protein in the AIF-silenced cells were a direct consequence of the increased levels of ROS.

Next, we addressed the question as to whether the increased ROS levels in AIF-silenced cells could participate in causing respiratory dysfunction,[20] a known outcome of ETC damage.[21] We measured whole-cell oxygen consumption rates in AIF-1-10 and AIF-2-4 and compared these to the pU6-2 and SURF-A7 control cells. At the same time, we also preincubated cells for 16 h in the presence of either 2.5 mM NAC[20] or 0.5 μM MitoQ, an antioxidant that is specifically targeted to the mitochondrion,[22] before measurement of respiration. The results, shown in Figure 1f, revealed that both AIF-1-10 and AIF-2-4 cells had an approximately 35% decreased rate of oxygen consumption (1.38 $\pm$ 0.03 nmol O_2 consumed/min/10^6 cells, $n=11$, $P<0.001$ and 1.59 $\pm$ 0.05, $n=6$, $P<0.05$, respectively) compared to pU6-2 (2.2 $\pm$ 0.08, $n=12$) and SURF-A7 (2.04 $\pm$ 0.06, $n=4$, data not shown). Interestingly, preculturing with either NAC or MitoQ could reverse the respiratory defect in AIF-silenced cells (AIF-1-10 plus NAC, 1.98 $\pm$ 0.1, $n=5$, $P<0.001$; AIF-1-10 plus MitoQ, 2.1 $\pm$ 0.1, $n=5$, $P<0.001$; AIF-2-4 plus NAC, 2.45 $\pm$ 0.05, $n=3$, $P<0.001$; AIF-2-4 plus MitoQ, 1.87 $\pm$ 0.07, $n=3$, $P<0.05$). Both NAC and MitoQ had no effect on basal O_2 consumption in pU6-2 (Figure 1f) or SURF-A7 (data not shown). To further understand the respiration defect in the AIF-silenced cells, we permeabilized a range of cell lines with digitonin and measured isolated Complex I-dependent respiration.[23] The results presented in Figure 1g demonstrate that whereas the control pU6-2 cells, respiring on the Complex I substrates malate (0.4 mM) and glutamate (30 mM), could be inhibited by nearly 90% with the Complex I inhibitor rotenone (6 μM), both AIF-silenced cell lines respired very poorly with these substrates and the rotenone-sensitive respiration was not different from respiration in the absence of the inhibitor. When succinate (10 mM), a Complex II electron donor, was added to cells, in order to bypass Complex I-dependent respiration, both AIF-silenced cell lines had O_2 consumption rates identical to control cell lines, suggesting that only Complex I is affected by AIF silencing. As anticipated, prior incubation with the mitochondrial antioxidant, MitoQ (0.5 μM), could restore normal levels of Complex I-dependent respiration in silenced cells, pointing to presumably ROS-mediated damage as the likely cause of the respiration deficiency. As recently described, the loss of AIF results in decreased abundance of Complex I subunit proteins.[13] In keeping with this observation, we performed Western blot analysis of our siAIF cell lines using an antibody directed to the Complex I protein p39, and confirmed that this subunit was significantly reduced (Figure 1h). As attempts to reverse the loss of this subunit in our model with antioxidants were unsuccessful (data not shown), we speculate that defects in Complex I could cause increased superoxide leakage, either by structural or stoichiometric alterations.[24] Alternatively, perhaps AIF acts on some other cellular component to maintain homeostatic ROS levels within the mitochondrion. Our future studies will be directed toward examining these possibilities.

In summary, our results show that silencing of the AIF gene results in increased free radical formation from the mitochondrion. These increases in ROS levels are physiologically relevant as (1) the ROS-sensitive transcription factor, HIF-1α, is stabilized and transcriptionally active and (2) O_2 consumption is perturbed at Complex I in the respiratory chain. Evidence that these processes are linked to free radicals arises from our findings that both outcomes can be reversed with antioxidant therapy. Our data, in part, are in agreement with a recent study that showed that AIF depletion resulted in decreased O_2 consumption, mainly at the site of Complex I.[13] As these authors could not detect changes in free radical levels in AIF-depleted cells, they raised the possibility that AIF could have a chaperone function in the mitochondrion, facilitating ETC component assembly. Our own findings would suggest that free radicals are increased upon AIF depletion, and are in line with a recent report suggesting that AIF may behave as a ROS scavenger.[9] Although the precise mechanism of AIF involvement in the ETC awaits further experimentation, data provided here strongly argue that free radicals play an important role in the observed respiration defect in AIF-silenced cells.

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