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Nicotinamide preferentially protects glycolysis in dermal fibroblasts under oxidative stress conditions

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Summary

Background Daily exposure of human skin to environmental insults such as solar radiation, pollution and smoke can lead to an elevation of oxidative stress, causing premature acceleration of skin ageing. Oxidative stress is known to disrupt cellular metabolism, which negatively impacts the skin's functionality at the cellular and tissue level.

Objectives To examine the changes in cellular metabolism due to oxidative stress.

Methods Glycolysis and oxidative phosphorylation rates in human dermal fibroblasts were monitored in real time under controlled nonlethal oxidative stress conditions. Hydrogen peroxide was utilized as a surrogate stressor because numerous environmental stressors as well as intrinsic ageing trigger its production.

Results Hydrogen peroxide ranging between 0·5 and 3 mmol L^{-1} caused a significant decrease in glycolytic and oxidative phosphorylation rates along with cellular ATP levels. Nicotinamide (NAM) was found to protect dose dependently as well as restore glycolytic rates concurrent with restoring ATP to control levels. NAM had an effective dose–response range between 0·1 and 1·0 mmol L^{-1}, with maximal effects attained at 0·5 mmol L^{-1}. Relative to oxidative phosphorylation, NAM was able to provide a diminished level of protection. FK866, a known NAM phosphoribosyltransferase inhibitor, was found to inhibit the protective effects of NAM significantly, suggesting part of the NAM mechanism of action involves nicotinamide adenine dinucleotide (NAD^+) synthesis.

Conclusions These results support previous findings that NAM protects cellular metabolism from oxidative stress by preferentially affecting glycolysis. Additionally, part of its mechanism of action appears to include NAD^+ synthesis.

One of the key functions that skin serves in human physiology is to provide protection from damaging environmental stresses that the body, particularly the face, experiences on a daily basis. These external stressors range from solar radiation to chemicals such as surfactants, to pollution such as fossil fuel exhaust, ozone or industrial particulates, as well as the mechanical and ambient climate. Understanding the molecular changes that occur in skin from both acute exposures and the long-term cumulative effects is important in order to identify intervention approaches that could potentially prevent and restore skin to a normal homeostatic state. It is thought that, of the numerous external stressors, ultraviolet (UV) radiation is the most significant driver of both acute and chronic changes in the skin. The effects of UV on the skin range from transient sunburn and reversible barrier damage to premature ageing/photoageing and to the more extreme condition of skin carcinomas such as melanoma. On a more molecular level, UV can cause macromolecule modifications such as nuclear and mitochondrial DNA (mtDNA) mutations/deletions, protein modification, lipid peroxidation, and cellular responses such as altered gene expression patterns, inflammatory infiltrate and production of reactive oxygen species (ROS) with concomitant oxidative stress.[1–3] Cumulative effects from acute exposure to UV lead to subsequent tissue changes such as erythema and chronic inflammation, elevated and inconsistent melanin pigmentation, skin thinning and loss in dermal matrix content and overall elasticity that can be histochemically and clinically measured.[4] Similar to internal organs, skin undergoes an intrinsic ageing process that can be mechanistically explained in part by the free radical theory first proposed in the 1950s.[5] The basis of this theory is that oxidative stress and subsequent cumulative damage from ROS

and free radicals are key drivers of the ageing process.[6] This is of relevance to human skin as elevated induction of oxidative stress and inflammation from UV and other environmental insults can lead to a further amplification of ROS and free radicals and thereby accelerate the free radical damage process, leading to photoageing.[7,8] One of the known negative effects of intrinsic ageing and oxidative stress on cells is altered and inefficient cellular metabolism.[9,10] Some of these changes in cellular metabolism include inhibition of metabolic enzyme activity,[11,12] altered mitochondrial efficiency, via, in part, mtDNA deletions,[9,13,14] and lowered energy metabolite production such as nicotinamide adenine dinucleotide (NAD^+).[15,16]

Nicotinamide (NAM) is a member of the vitamin B family (B_3, niacinamide) and serves as a precursor for synthesis of NAD.[17,18] Over the past few decades, research on NAM has shown that it can significantly mitigate some of the acute and chronic effects of UV exposure on the skin. Based on *in vitro* and *in vivo* studies, it is hypothesized that one of the primary modes of action of NAM is incorporation into the NAD^+ pool[17–20] and facilitating metabolism and ATP synthesis.[21] More recently it has been shown that NAD^+ serves an important function as a substrate for poly(adenosine diphosphate ribose) polymerase (PARP)-1 enzymatic activity to repair UV-induced DNA mutations/deletions[22] as well as for sirtuins that regulate metabolism via selective acetylation of metabolic enzymes.[23] NAD^+ levels can be lowered acutely by UV exposure of the skin,[15] as well as due to intrinsic ageing as measured in photoprotected skin sites.[24] Topical application of NAM has shown its ability to reverse and repair some of the negative effects induced by photoageing and UV exposure, as well as other skin conditions such as acne and rosacea.[25–27] Both topical and oral NAM have been shown to prevent UV-induced immunosuppression,[28,29] a response of skin to UV exposure that is thought to be involved in development of skin carcinomas.[30] Microarray analysis of NAM effects on UV-induced immunosuppression has led to the mechanistic hypothesis that part of its protective effects involves protection of cellular metabolism,[29] including both oxidative phosphorylation and glycolysis.[31,32]

Based on this, we wished to monitor the protective effects of NAM on glycolysis and oxidative phosphorylation in real time under conditions of oxidative stress and, additionally, to determine whether NAM is able to restore or repair damage to these two metabolic processes. FK866, a selective inhibitor of NAM phosphoribosyltransferase (NAMPT),[33] was utilized to determine whether NAD^+ synthesis is a component of the NAM mechanism of action.

Materials and methods

Cells and reagents

Human neonatal BJ fibroblasts were purchased from the American Tissue Culture Collection (ATTC, Manassas, VA, U.S.A.). Cells were grown in Eagle's minimum essential medium (ATCC) supplemented with 10% fetal bovine serum (ATCC) and gentamicin/amphotericin B × 500 solution (Invitrogen, San Diego, CA, U.S.A.) in T-150 flasks (Corning Inc., Corning, NY, U.S.A.). Cells were cultured at 37 °C, 5% CO_2 and 90% relative humidity. FK866 was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). All other reagents were purchased from Sigma Aldrich (St Louis, MO, U.S.A.).

Measurement of glycolysis and oxidative phosphorylation

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measurements from dermal fibroblasts were made using an XF24 and XF96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, U.S.A.). Experiments were conducted with a sensor cartridge and 24- or 96-well V7 plates (Seahorse Bioscience) coated with 0·2% gelatin. The sensor cartridge was hydrated overnight with the addition of calibrant (Seahorse Bioscience; 1 mL for 24-well and 200 μL for 96-well plates) and incubated overnight at 37 °C without CO_2. Gelatin and PBS were warmed to 37 °C. Gelatin (50 μL for 24-well and 20 μL for 96-well plates) diluted 1: 10–0·2% in sterile PBS was added to each well and incubated at room temperature for 30 min. After 30 min, excess liquid was removed by aspiration without touching the surface of the wells. The plates were then incubated at room temperature to dry the gelatin for at least 2 h. Cells were incubated in a T-150 flask to 80% confluence. Dermal fibroblasts were trypsinized from the T-150 flask and plated at 1×10^5 per well for 24 wells and 4×10^4 for 96 wells in 100 μL of growth medium in gelatin-coated Seahorse Bioscience V7 plates 24 h prior to the experiment.

Prior to measurement of OCR and ECAR, growth medium was removed and for 24-well plates the cells were washed with 1·0 mL Dulbecco's modified Eagle's medium (Seahorse Bioscience) containing 25 mmol L^{-1} glucose and 1 mmol L^{-1} pyruvate warmed to 37 °C at pH 7·4 and replaced with 600 μL of assay medium. For the 96-well plates, cells were washed and medium replaced via the Prep Station (Seahorse Bioscience) per the manufacturer's instructions using the medium described above. Cells were incubated at 37 °C in a CO_2-free incubator for 1 h prior to measurements.

H_2O_2 was utilized to cause oxidative damage and was prepared at 10× working concentration in assay medium (pH 7·4), warmed to 37 °C, and loaded into an injection port of the sensor cartridge prior to calibration. NAM and FK866 were prepared at 10× working concentration in assay medium (pH 7·4), warmed to 37 °C, and loaded into the injection port prior to calibration. Final solutions were adjusted to pH 7·4 if necessary. Sensor cartridges were loaded into the XF24 Analyzer per the manufacturer's instructions; cells were loaded into the XF24 following the manufacturer's instructions and further equilibrated prior to the first measurement. Experiments were performed at 37 °C. A minimum of three separate experiments was performed and data analysed from either three replicates from XF 24-well plates or eight

replicates from XF 96-well plates. Statistical analysis was performed on either the last time point from the trace or the area under the curve when appropriate. Student's t-test was used for statistical analysis with data presented as mean value and standard deviation.

ATP measurement

ATP was quantified from cells in culture using the ATP Cell Titer Glo® assay (Promega, Madison, WI, U.S.A.). The medium was removed from cells, washed with 200 μL PBS and replaced with 100 μL PBS to each well. Cell Titer Glo reagent was prepared according to the manufacturer's instructions and 100 μL was added to cells. Cells were incubated with reagent according to the manufacturer's instructions and 200 μL transferred from the 24-well V7 plate to a black, clear-bottom, 96-well plate in order to quantify ATP via luminescence. A minimum of three separate experiments was performed and data analysed either from three replicates from XF24-well plates or eight replicates from XF96-well plates. Student's t-test was used for statistical analysis with data presented as mean value and standard deviation.

Results

Nicotinamide preferentially blocks hydrogen peroxide-induced decreases in glycolytic rates

To monitor the effects of hydrogen peroxide-induced oxidative stress on cellular metabolism, BJ dermal fibroblasts were exposed to 0·1–3·0 mmol L^{-1} hydrogen peroxide to determine optimal and reproducible nonlethal conditions while maintaining cellular viability and integrity. Glycolysis and oxidative phosphorylation rates were monitored in real time using either the 24-well or 96-well Flux Analyzer. Glycolysis measurements are reported as ECAR and oxidative phosphorylation as OCR. Hydrogen peroxide dose–response curves showed that hydrogen peroxide concentrations of $\geq$ 0·5 mmol L^{-1} were required to impact cellular metabolism significantly. Hydrogen peroxide at 0·5 mmol L^{-1} showed a significant increase in ECAR with a compensatory response in decreased OCR (Fig. 1). A significant decrease in ECAR was observed 30 min after a cumulative concentration of 1·5 mmol L^{-1} hydrogen peroxide was attained (Fig. 1a, third arrow, $P = 0{\cdot}02$ for last time point) and this decrease was significantly prevented by the presence of 1 mmol L^{-1} NAM ($P = 0{\cdot}009$). However, there was no apparent effect from NAM on protecting OCR under these conditions (Fig. 1b). Analysis of cellular ATP levels at the last time point showed an 80% decrease, and 1 mmol L^{-1} NAM was able significantly to maintain levels at the control medium levels (Fig. 1c). Separately, to determine whether exposure to 1·5 mmol L^{-1} hydrogen peroxide during this time course was lethal to fibroblasts, assessments of cellular integrity were performed. Fibroblasts were exposed to 1·5 mmol L^{-1} hydrogen peroxide for 90 min, switched to fresh medium with no peroxide, incubated for an additional 1 and 24 h, and assayed for cellular ATP and protein content. One hour after peroxide exposure there was a similar 80% decrease in cellular ATP levels but with no significant change in total cellular protein. Cellular ATP levels returned to basal control levels 24 h after peroxide exposure, suggesting that the acute exposure was not lethal. This was supported by visualization of overall cellular morphology (data not shown). To test further whether NAM can protect cellular metabolism at higher concentrations of hydrogen peroxide, a similar injection sequence was performed with 1 mmol L^{-1} hydrogen peroxide. Exposure of cells to 1 mmol L^{-1} NAM at the first hydrogen peroxide injection showed an ability significantly to maintain ECAR at control levels but not OCR (Fig. 1d,e, first arrow). A cumulative concentration of 2 mmol L^{-1} hydrogen peroxide led to a significant decrease in both ECAR and OCR (Fig. 1d, e, second arrow). Attaining 3 mmol L^{-1} hydrogen peroxide led to a further decline in ECAR and OCR (Fig. 1d,e, third arrow) along with a 93% decrease in cellular ATP levels (Fig. 1f). The presence of 1 mmol L^{-1} NAM throughout all three injections significantly protected ECAR ($P < 0{\cdot}0001$) to nearly control levels but not OCR during the course of the experiment with the exception at the last time point (minute 199). This late effect of NAM on OCR was a consistent observation over a minimum of three repeated experiments. In contrast to exposure to 1·5 mmol L^{-1} hydrogen peroxide, 1 mmol L^{-1} NAM maintained cellular ATP levels at 35% of control when cells were exposed to 3 mmol L^{-1} hydrogen peroxide (Fig. 1f).

Dose–response effect of nicotinamide protection on glycolysis but not oxidative phosphorylation

To determine whether the protective effect of NAM on cellular metabolism was dose sensitive, fibroblasts were treated with a single injection of 1·5 mmol L^{-1} hydrogen peroxide and a 0·1–1 mmol L^{-1} concentration range of NAM and monitored for 56 min after treatment. The effects of increasing NAM on ECAR recorded at the last time point showed a significant increase in protection up to 0·5 mmol L^{-1} (Fig. 2a). The protective effect on OCR by NAM as typically observed at the last time point was significant ($P = 0{\cdot}001$ or lower) but not dose responsive (Fig. 2b). Cellular ATP levels also showed a significant dose-responsive effect from 0·1 mmol L^{-1} NAM ($P = 0{\cdot}005$) with an inflection point at 0·5 mmol L^{-1} NAM (Fig. 2c; $P < 0{\cdot}001$).

Maximizing nicotinamide protection on oxidative phosphorylation

Fibroblast cells were exposed to 1·5 mmol L^{-1} hydrogen peroxide and a sequential injection of NAM up to 3 mmol L^{-1} final concentration to determine whether protection of OCR by NAM can occur at higher concentrations. ECAR remained the same between 1 and 3 mmol L^{-1} NAM, with both remaining at control levels as previously observed (data not

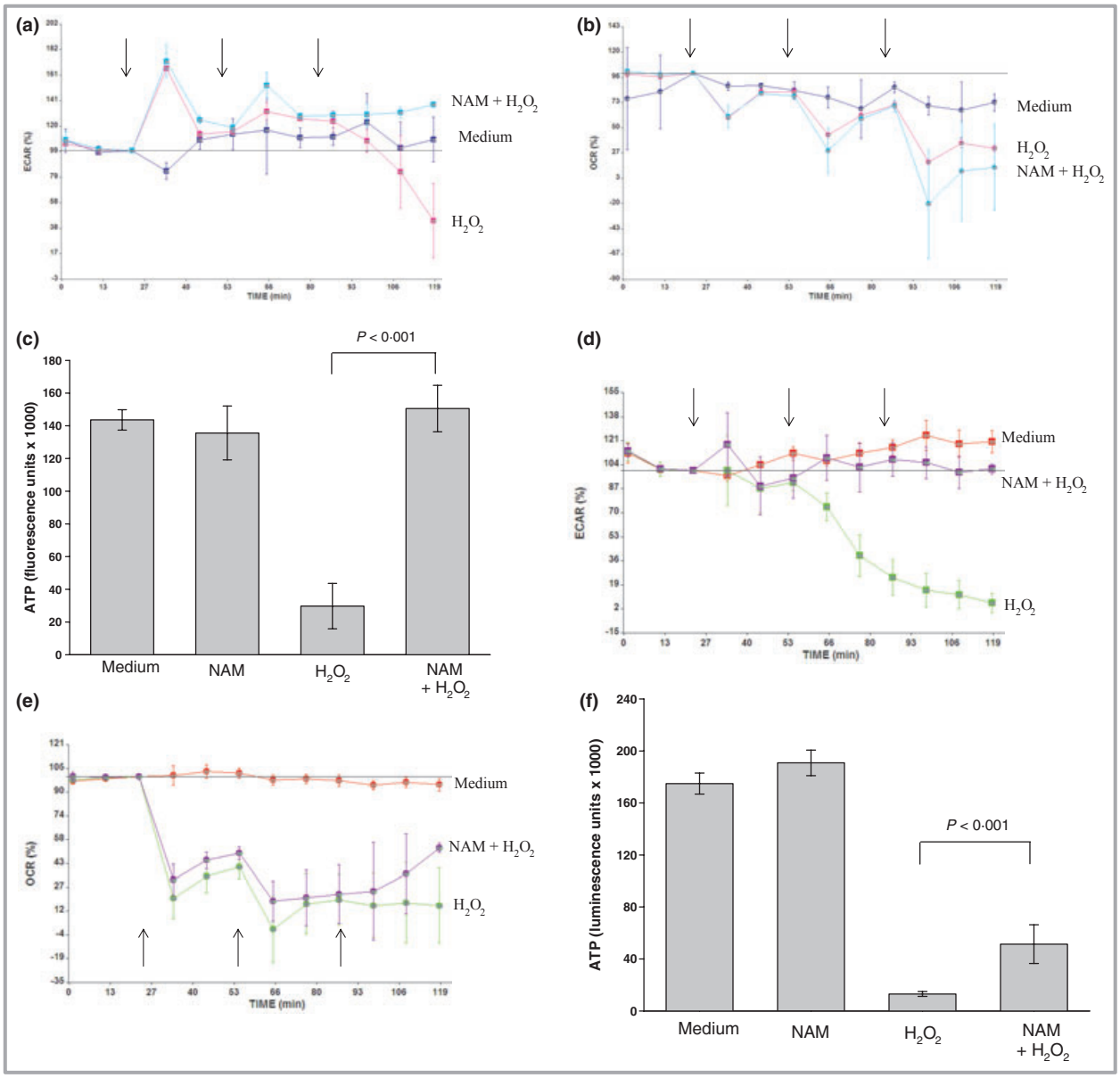

Fig 1. Glycolytic protection of hydrogen peroxide-induced oxidative stress by nicotinamide (NAM). Cellular respiration measured by the XF Flux Analyzer (see text) shown as (a) extracellular acidification rate (ECAR) and (b) oxygen consumption rate (OCR) of oxidative stress in dermal fibroblasts induced by three injections of 0·5 mmol L^{-1} H_2O_2 (arrows) over time and treated with one injection of 1 mmol L^{-1} NAM (first arrow). (c) Quantification of ATP was completed after final cellular respiration measurement. (d) ECAR and (e) OCR of measurement of higher concentration of H_2O_2 by three injections of 1·0 mmol L^{-1} H_2O_2 (arrows) and one injection of 1 mmol L^{-1} NAM (first arrow). (f) ATP quantification following three injections of 1·0 mmol L^{-1} H_2O_2 and one injection of 1·0 mmol L^{-1} NAM.

shown). While there was a significant difference at the last time point between hydrogen peroxide treatment and both 1 and 3 mmol L^{-1} NAM, neither NAM treatment was able to return levels to those of the control. There was no significant difference in OCR between 1 and 3 mmol L^{-1} NAM up to 90 min after oxidative stress nor at the last time point (Fig. 3a,b). Quantification of cellular ATP levels at the end of the time course showed a significant effect by 3 mmol L^{-1} NAM on restoring levels to control levels in comparison to 1 mmol L^{-1} NAM (Fig. 3c).

Nicotinamide pretreatment effect on protecting glycolysis and oxidative phosphorylation

Fibroblasts were treated with 1 mmol L^{-1} NAM overnight prior to oxidative stress induction with hydrogen peroxide to determine whether there was any assimilation of protective effects from NAM. Pretreatment with NAM did not lead to any significant protective effects of either ECAR or OCR over several experiments (data not shown). A range of hydrogen peroxide exposures was used to determine whether pretreat-

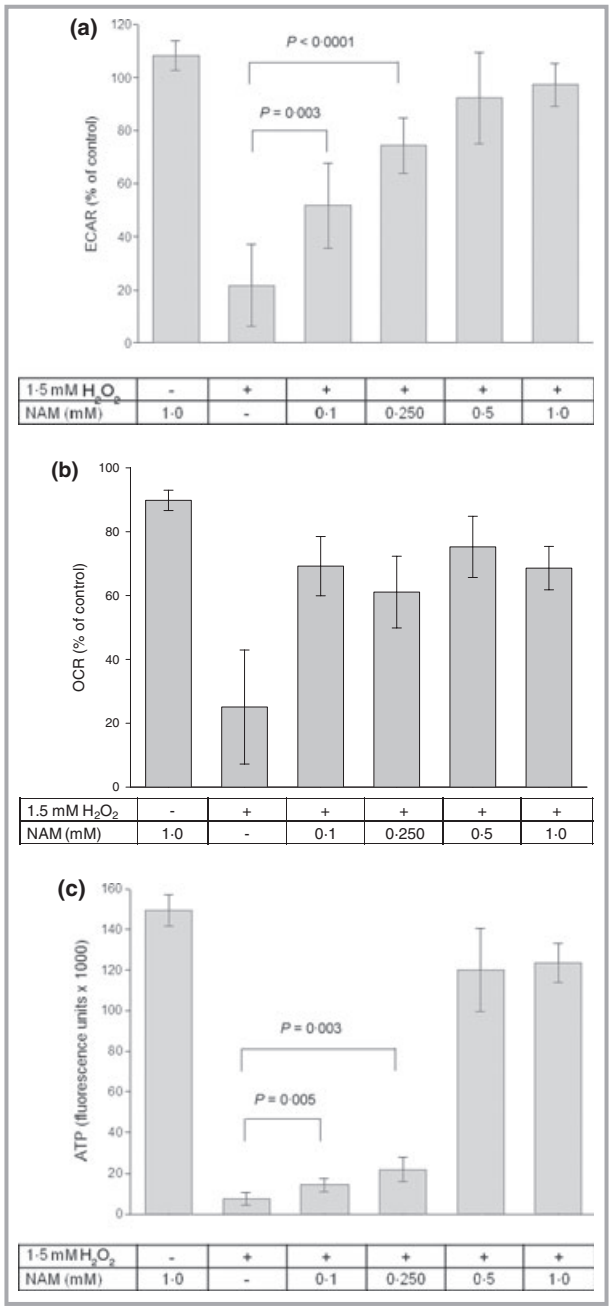

Fig 2. Nicotinamide (NAM) dose-dependent glycolytic protection from hydrogen peroxide. Oxidative stress was induced by 1·5 mmol L^{-1} H_2O_2 and increasing concentrations of NAM were co-injected; cellular respiration was evaluated over time using the XF Flux Analyzer (see text), shown as (a) extracellular acidification rate (ECAR) and (b) oxygen consumption rate (OCR) represented 56 min after injection. (c) Following the last measurement of cellular respiration ATP was quantified.

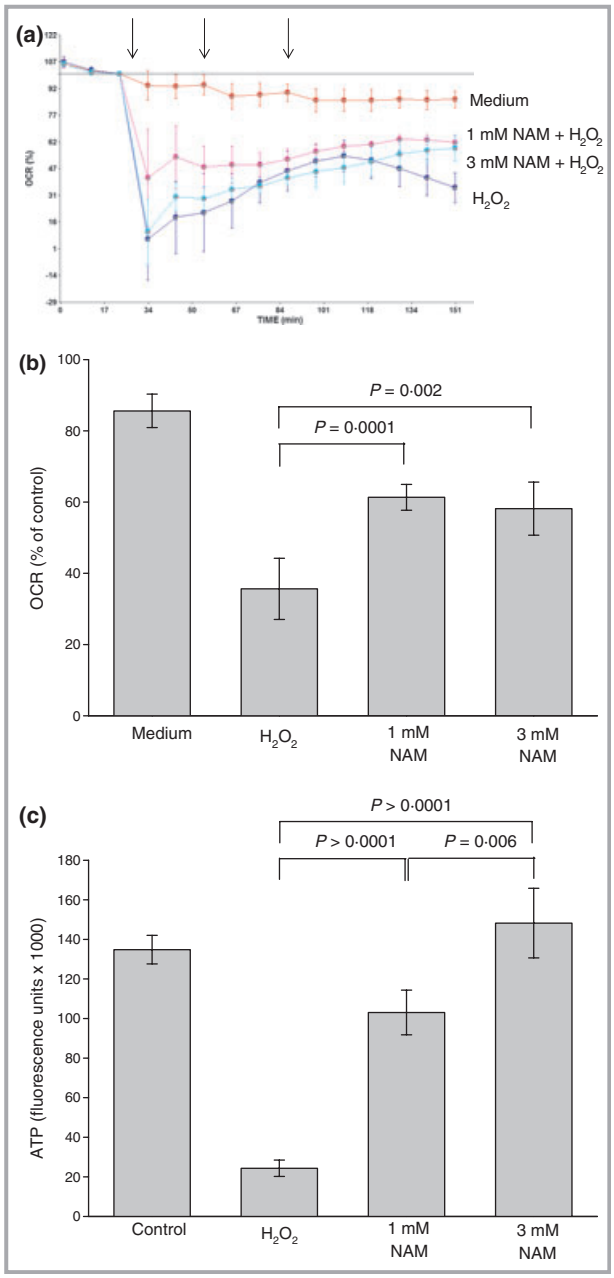

Fig 3. Increasing nicotinamide (NAM) concentration to protect oxidative phosphorylation. Dermal fibroblasts were stressed with one injection of 1·5 mmol L^{-1} H_2O_2 (first arrow) and co-treated with one or three injections of 1·0 mmol L^{-1} NAM (arrows) over time; cellular respiration was evaluated using the XF Flux Analyzer (see text). (a) Extracellular acidification rate (ECAR) shown as a trace over time and (b) oxygen consumption rate (OCR) represented 90 min after the first injection. (c) ATP was quantified following analysis of cellular respiration.

ment could add in combination to any protective effects from co-treatment of NAM. Reduction in ECAR and loss of cellular ATP levels with 1·5 mmol L^{-1} hydrogen peroxide could be completely reversed with 1 mmol L^{-1} NAM as previously determined (data not shown). However, overnight pretreatment of fibroblasts with 1 mmol L^{-1} NAM followed by co-treatment during 1·5 mmol L^{-1} hydrogen peroxide exposure had no statistically significant effect on protecting OCR (Fig. 4a) nor on cellular ATP levels (data not shown).

To assess further any pretreatment effects on ECAR, 3 mmol L^{-1} hydrogen peroxide was used to induce oxidative

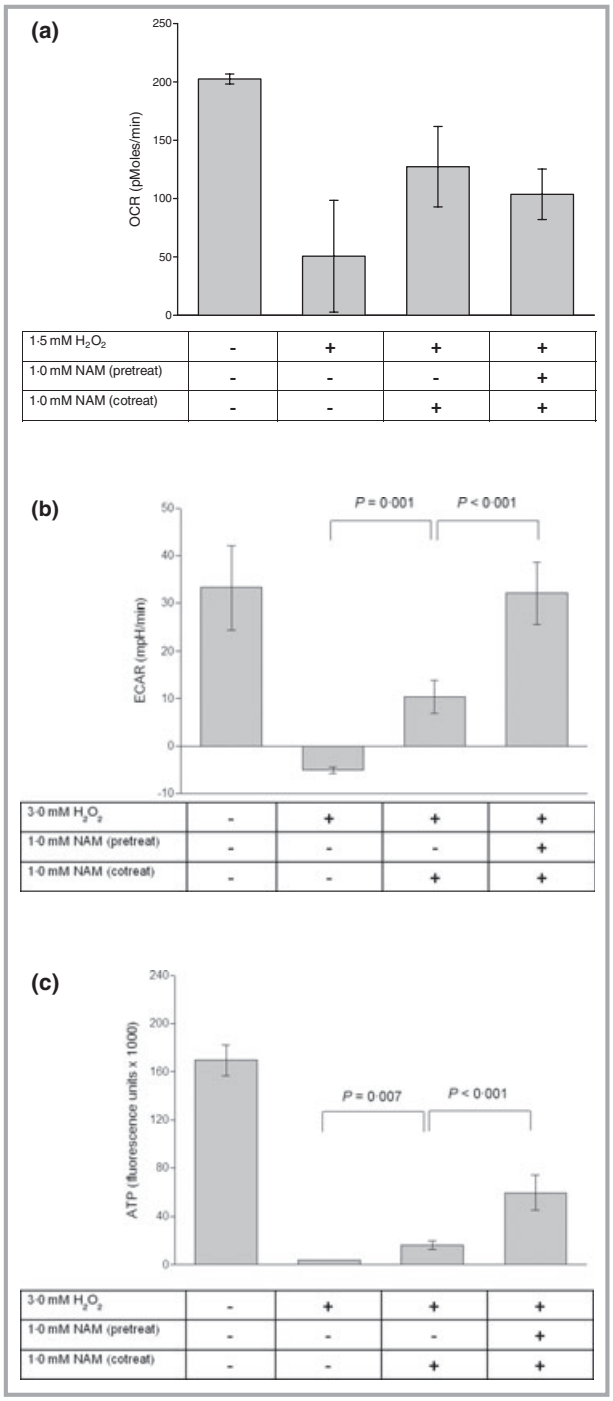

Fig 4. Pretreatment with nicotinamide (NAM) to protect stress-induced damage of glycolysis and oxidative phosphorylation. Dermal fibroblasts were pretreated overnight with 1·0 mmol L^{-1} NAM. (a) Cells were pretreated overnight and stressed the following day with three injections of 0·5 mmol L^{-1} H_2O_2 over time with a selection of wells co-treated with 1·0 mmol L^{-1} NAM. Data represents 84 min after the first injection. (b) Extracellular acidification rate (ECAR) and (c) ATP of cells pretreated overnight with 1·0 mmol L^{-1} NAM and stressed the following day with three injections of 1·0 mmol L^{-1} H_2O_2 over time plus a selection of wells co-treated with 1·0 mmol L^{-1} NAM. Data shown are 84 min after the first injection. OCR, oxygen consumption rate; mpH, milli-pH.

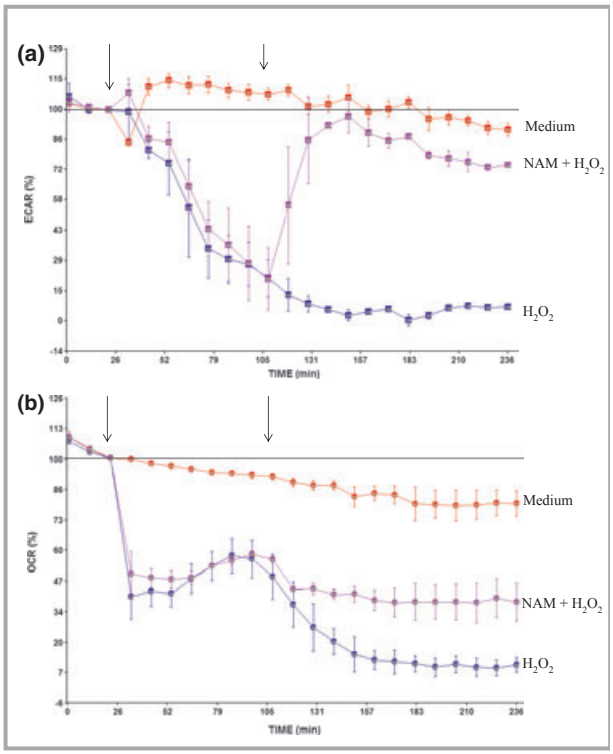

Fig 5. Reversal by nicotinamide (NAM) of hydrogen peroxide-induced oxidative stress. Dermal fibroblasts were stressed with 1·5 mmol L^{-1} H_2O_2 (first arrow), incubated for 86 min and treated with 1·0 mmol L^{-1} NAM (second arrow). (a) Extracellular acidification rate (ECAR) and (b) oxygen consumption rate (OCR) were measured using the XF Flux Analyzer (see text).

stress on cellular metabolism. Co-treatment of 1 mmol L^{-1} NAM with 3 mmol L^{-1} hydrogen peroxide resulted in a lower level of protection of ECAR as well as cellular ATP levels (Fig. 4b,c, respectively). Pretreatment of fibroblasts overnight with 1 mmol L^{-1} NAM followed by co-treatment with 1 mmol L^{-1} NAM during 3 mmol L^{-1} hydrogen peroxide exposure led to a significant increase in protective effects on ECAR along with an increase in cellular ATP levels (Fig. 4b,c, respectively). There were no significant protective effects on OCR under either NAM treatment sequence (data not shown).

Nicotinamide can reverse hydrogen peroxide-induced stress on glycolysis and oxidative phosphorylation

While NAM was shown to protect glycolysis and oxidative phosphorylation to a lesser extent from hydrogen peroxide-induced oxidative stress, we wished to assess whether NAM is able to restore both metabolic processes after induction of the stress and damage. As previously described, fibroblasts were exposed for 56 min to 1·5 mmol L^{-1} hydrogen peroxide to induce oxidative stress and reduce ECAR and OCR. Subsequent injection of 1 mmol L^{-1} NAM led to a significant reversal of decreased ECAR compared with 1·5 mmol L^{-1} hydrogen peroxide and to within 83% of control levels (Fig. 5a; $P < 0·001$ at last time point). NAM was also able to restore OCR but

only to within 29% of control levels (Fig. 5b; $P < 0{\cdot}001$ at last time point).

To test further the ability of NAM to reverse damage to ECAR and OCR after oxidative stress, we assessed the effects of NAM on ECAR and OCR after removal of hydrogen peroxide. Fibroblasts were exposed to 1·5 mmol L^{-1} hydrogen peroxide for 56 min to reduce significantly both ECAR and OCR as previously determined (data not shown). Cells were then washed with fresh medium to remove hydrogen peroxide and cells were incubated back in the Flux Analyzer with fresh medium. NAM (1 mmol L^{-1}) injected 21 min after equilibration showed a significant effect ($P < 0{\cdot}001$ on last time point) on increasing ECAR to 55% of control levels (Fig. 6a). NAM was also able partially to restore OCR ($P = 0{\cdot}002$ on last time point) to within 37% of control levels (Fig. 6b). Treatment with medium alone after peroxide stress showed a reversal in decreased ECAR and OCR to 10% and 13% of control levels, respectively. These effects correlated with a significant increase in ATP compared with hydrogen peroxide medium-treated cells and was 17% of control levels (Fig. 6c).

FK866 significantly reduces nicotinamide protective effects of glycolysis

As it is well known that NAM functions in a large part as a precursor in the salvage pathway for synthesis of NAD^+, we wished to assess whether the NAM mechanism of action involves this enzymatic process. The NAMPT inhibitor FK866 was tested at nontoxic levels both alone and in combination with NAM under hydrogen peroxide stress conditions. Fibroblasts were plated under standard conditions and then exposed either to medium alone or to 10 and 25 μmol L^{-1} FK866 for 30 min. Cells were then exposed to 1·5 mmol L^{-1} hydrogen peroxide stress conditions with and without 0·25 mmol L^{-1} NAM. A lower concentration of NAM was used to ensure more direct competitive comparison between it and FK866 for NAMPT active site interaction. An additional 90 min of monitoring showed that FK866 can inhibit the recovery effect of NAM on ECAR (Fig. 7a). FK866 at 25 μmol L^{-1} showed a significant effect on reducing NAM protection of glycolysis by 60% (Fig. 7b) whereas 10 μmol L^{-1} FK866 had no significant effect (data not shown). There was no apparent effect from 25 μmol L^{-1} FK866 alone on ECAR (Fig. 7b). There was no significant effect at either concentration of FK866 on NAM protection of OCR (data not shown).

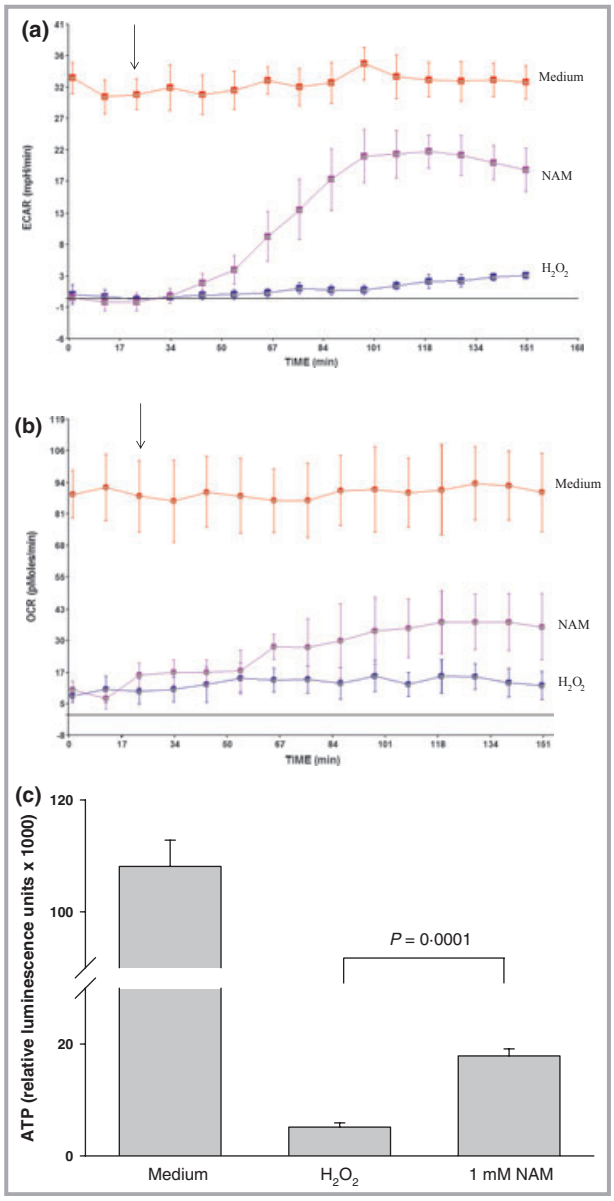

Fig 6. Evaluation of nicotinamide (NAM) for reversing oxidative stress after removal of hydrogen peroxide. Dermal fibroblasts were stressed with 1·5 mmol L^{-1} H_2O_2 and incubated for 75 min. Medium was removed and cells were washed with assay medium and further incubated to show damage, then injected with 1·0 mmol L^{-1} NAM (arrow). (a) Extracellular acidification rate (ECAR) and (b) oxygen consumption rate (OCR) were measured using the XF Flux Analyzer (see text); (c) ATP was quantified after the last time point. mpH, milli-pH.

Discussion

Exposure of human skin to environmental stressors leads to a range of changes that includes oxidative stress. Oxidative stress causes changes in cellular metabolism capacity that is hypothesized to be a critical component in causing cellular dysfunction. The cumulative effect from daily insults, particularly on the face, causes the premature ageing phenomenon known as photoageing and can also lead to the more serious condition of skin carcinoma induction. This work provides additional understanding on NAM ability to block critical cellular bioenergetic processes under conditions of oxidative stress. We report here that NAM has the ability to protect and restore glycolysis dose dependently in dermal fibroblasts under oxidative stress. NAM was able partially to protect oxidative phosphorylation but not in a dose-dependent manner nor was it able to restore to control levels after oxidative damage, suggesting a preferential protection of glycolysis. Inhibition by

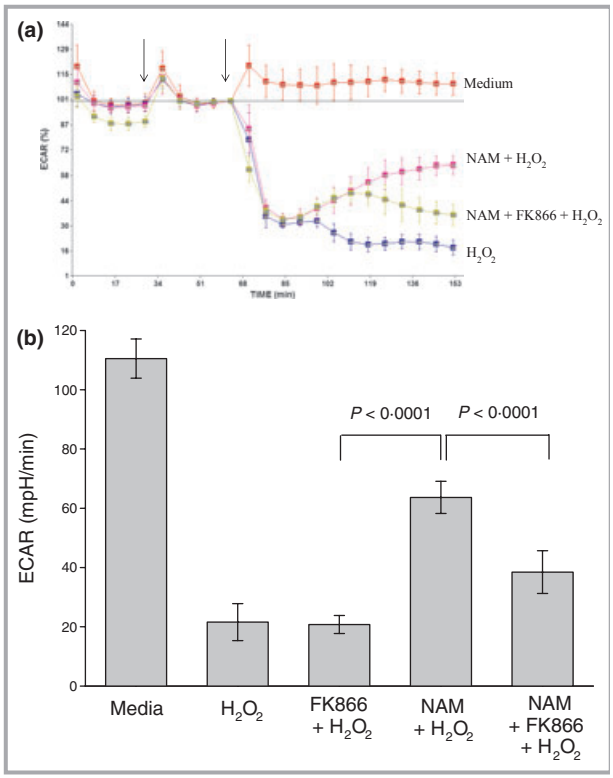

Fig 7. Nicotinamide (NAM) protection of glycolysis was reduced with FK866 co-treatment. Dermal fibroblasts were pretreated with 10 μmol L^{-1} FK866 (first arrow) and co-treated with 0·25 mmol L^{-1} of NAM and stressed with 1·5 mmol L^{-1} H_2O_2 (second arrow). Extracellular acidification rate (ECAR) was measured over time and is represented as a trace (a) and the final time point measurement is shown as a bar graph (b). mpH, milli-pH.

FK866 of NAM protective effects on glycolysis implies that synthesis of NAD^+ is a critical part of its mechanism of action.

As NAM has been known to protect cellular metabolism in skin and cells exposed to UV,[31] we assessed both glycolysis and oxidative phosphorylation in real time to determine more closely its dose–response profile and differential effects on these cellular bioenergetic pathways. Hydrogen peroxide was used to induce oxidative stress in the *in vitro* system presented here based on it being a common 'denominator' of the skin's response to such insults as UV radiation, cigarette smoke, and pollution as well as intrinsic ageing, as it relates to the free radical theory of ageing.[34–36] Under real-life conditions human skin, particularly the face, is exposed on a daily basis to multiple environmental stressors simultaneously, which can lead to amplified ROS production and heightened levels of damage and oxidative stress.[37,38] The specific conditions utilized in this work were selected based on concentrations that induced measureable and significant decreases in glycolytic and oxidative phosphorylation rates and cellular ATP pools. Conditions were selected that were determined to be nonlethal and reversible based on the ability of BJ dermal fibroblasts to recover to control cellular viability levels as determined by ATP levels, total protein amount and cellular morphology visualization after exposure to 1·5 mmol L^{-1} hydrogen peroxide (data not shown). It should be noted that BJ dermal fibroblasts have a greater tolerance to oxidative stress than normal dermal fibroblasts due to a high antioxidative capacity.[39] It is not believed that the observed NAM effects are based on quenching or chemical interaction with peroxide nor subsequent generated free radicals. Spectrophotometric monitoring of the NAM absorption profile in the presence of 3 mmol L^{-1} hydrogen peroxide did not show any significant loss of absorption nor novel absorption peaks or shifts (data not shown). The ability of NAM partially to restore glycolysis after removal of hydrogen peroxide and inhibition by FK866 at low micromolar concentrations further supports the theory that the effects reported here were due to a biochemical-based reaction.

Nicotinamide (NAM) protection of cellular bioenergetics showed a differential preference for glycolysis over oxidative phosphorylation. Cells treated with hydrogen peroxide up to 1·0 mmol L^{-1} displayed elevated glycolytic rates, presumably to offset metabolically lower oxidative phosphorylation rates. At 1·5 mmol L^{-1} hydrogen peroxide, both metabolic processes were negatively impacted and NAM showed a consistent and significant ability to protect against this oxidative stress response and maintain glycolysis at control levels. Additionally, it was able to restore glycolysis to control levels after initial induction of damage but was not able completely to reverse the damage after removal of hydrogen peroxide. At elevated levels of stress with 3 mmol L^{-1} hydrogen peroxide, NAM protected glycolysis but protection of oxidative phosphorylation was significantly lower. This preferential protection of glycolysis may be due in part to cellular compartmentalization and NAM metabolism,[19] supported by our findings that FK866 can reduce the NAM protective effect by 60%. This difference in protection and partial inhibition by FK866 could be explained in part by cellular location of NAD^+ pools. FK866 has been shown to reduce cytoplasmic NAD pools but not mitochondrial pools due to the absence of the NAD salvage pathway in mitochondria.[40] The absence of the NAD salvage pathway is supported by findings that NAM does not directly incorporate into mitochondrial NAD pools and requires conversion to the intermediate, nicotinamide mononucleotide (NMN), by NAMPT in the cytoplasm. NMN is subsequently transported into mitochondria where it can be converted to NAD by NMN adenylyl transferase (NMNAT) in mitochondria.[41] Thus, it is hypothesized that NAM has a more direct impact on glycolysis via rapid incorporation into cytoplasmic NAD^+ pools. Additionally, it has been reported that NAM protective effects on glycolysis and ATP levels from UV damage are due in part to inhibition of PARP.[32] However, it was suggested that the effect could not be explained fully by this mechanism and that incorporation into the NAD^+ pool could be involved. We show here that the NAMPT inhibitor, FK866, is able to reduce significantly the protective effects of NAM, supporting the finding that NAD^+ synthesis is part of the NAM mechanism of action. Because the protective effects were not completely blocked by FK866, it stands to reason

that both NAD^+ synthesis and PARP inhibition are components of the NAM mechanism of action for protecting glycolysis, and to a lower extent, oxidative phosphorylation.

The results presented here continue to show the important role of NAM in maintaining cellular homeostasis and in particular metabolism. NAM and NAD^+ are important regulators of metabolism and mitochondrial autophagy, signalling cascades, cellular response to oxidative stress, and ageing continues to evolve.[42–46] Additionally, there are potential health implications in understanding the mechanism of action in blocking UV-induced oxidative stress. Continued studies assessing NAM effects on protecting glycolysis and oxidative phosphorylation in real time under other environmental stresses such as UV and cigarette smoke are merited. As NAM and NAD^+ are inhibitors and substrates, respectively, for both PARP and sirtuins, it is important to explore further the interplay between regulation of cellular metabolism and DNA repair in response to environmental stressors and ageing.

What's already known about this topic?

- Oxidative stress of dermal fibroblasts leads to a decrease in cellular metabolism.
- Nicotinamide mitigates ultraviolet-induced immunosuppression by protecting cellular metabolism.
- Acute hydrogen peroxide-induced oxidative stress leads to decreases in glycolytic and oxidative phosphorylation rates correlating with a transient decrease in ATP levels.

What does this study add?

- Nicotinamide is able to protect and partially restore glycolytic rates to nonstressed levels. In contrast, nicotinamide is only partially able to restore oxidative phosphorylation rates and not to protect them after oxidative stress. These effects correlate with maintaining and restoring cellular ATP levels to varying levels.
- FK866, a nicotinamide phosphoribosyltransferase inhibitor, significantly reduces the protective effect of nicotinamide on glycolysis and oxidative phosphorylation, suggesting incorporation into the cellular NAD^+ pool is an important component of the mechanism of action of nicotinamide.

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