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The anti-inflammatory and antioxidative effects of nicotinamide, a vitamin B_3 derivative, are elicited by FoxO3 in human gestational tissues: implications for preterm birth☆,☆☆

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Abstract

The inflammatory process plays a pivotal role during the pathogenesis of human labour, both at term and at preterm. Nicotinamide, a vitamin B_3 derivative, exerts anti-inflammatory and antioxidative properties in several cell types by interaction with various intracellular signalling proteins via modulating the activity of various transcription factors, including activation of the O subfamily of Forkhead/winged helix transcription factors (FoxO) and inhibition of nuclear factor-κB (NF-κB). The aim of this study was to determine the effect of nicotinamide on the expression of pro-labour and mediators in human placenta. The effects of nicotinamide were evaluated, over 24 h, by treating placenta with 0, 25 and 50 mM nicotinamide in the absence or presence of 10 μg/ml lipopolysaccharide (LPS). Nicotinamide treatment resulted in a significant reduction of basal and/or LPS-stimulated release and gene expression of the pro-inflammatory cytokines TNF-α, IL-6 and the chemokine IL-8, and the release of the prostaglandins PGE_2 and $PGF_2\alpha$ and cyclooxygenase (COX)-2 mRNA expression. Additionally, nicotinamide treatment of human placenta resulted in attenuation of basal and LPS-induced oxidative stress, reducing 8-isoprostane release and increasing gene expression of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). There was no effect of nicotinamide on NF-κB activation. The anti-inflammatory and antioxidant actions of nicotinamide were abolished by knockdown of FoxO3 using siRNA. In conclusion, nicotinamide exerts anti-inflammatory and antioxidative effects in human placenta, in part, via activation of FoxO3. Further studies should be undertaken to define a possible implication of vitamin B_3 derivatives in the management of preterm labour and delivery.

Keywords: Nicotinamide; Vitamin B_3; Cytokines; Prostaglandins; Oxidative stress; FoxO3; Parturition

1. Introduction

The cellular and molecular mechanisms that govern birth are not fully elucidated. This lack of understanding limits our ability to reduce the incidence of labour complications, including preterm labour and delivery, which is the single most important complication contributing to poor pregnancy and neonatal outcome [1]. Together with the fiscal costs of caring for the affected infants in neonatal intensive care units, these deficits impose a serious burden on health systems, families and society at large [2].

For the successful outcome to labour and delivery, fetal maturation, cervical ripening and dilatation, uterine contractions and fetal membrane rupture are required. Reactive oxygen species (ROS), inflammatory mediators (e.g., pro-inflammatory cytokines), uterotonic phospholipid metabolites (e.g. prostaglandins) and the induction of extracellular matrix (ECM) remodelling play a central role in these terminal pathways of human labour, both at term and at preterm and at delivery [3-8]. Thus, the key to improving the management of preterm birth is an understanding of how the multiple processes that are requisite for a successful labour and delivery are coordinated to achieve a timely birth.

Nicotinamide, also known as niacin, niacinamide and nicotinic acid amide, is the amide of nicotinic acid (vitamin B_3). Vitamin B_3 is found in nuts, dairy products, lean meats, poultry, fish, eggs, seeds, legumes and enriched breads and cereals. Milk, green leafy vegetables, coffee and tea also provide some niacin. Recently, interest in nicotinamide has shifted from its role as a nutrient to that as a novel pharmacological agent. Nicotinamide, via its major metabolite NAD++ (nicotinamide adenine dinucleotide), is involved in a wide range of biological

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processes including the production of energy, the synthesis of fatty acids, cholesterol and steroids, signal transduction and the maintenance of the integrity of the genome [9,10]. Of importance to this study, nicotinamide has anti-inflammatory [11-20] and antioxidant [21-24] properties. Nicotinamide relies upon unique cellular pathways that involve the O subfamily of Forkhead/winged helix transcription factors (FoxO), enzyme silent information regulator 2/sirtuin 1 (SIRT1) and protein kinase B (Akt) [9,10,25]. Furthermore, a recent study has shown that nicotinamide inhibits pro-inflammatory mediators through nuclear factor-κB (NF-κB) [18], a key regulator of pro-labour mediators in human gestational tissues [26-32].

The effect of nicotinamide in human gestational tissues as modulators of the inflammatory response that is associated with human labour is not yet known. Therefore, the objective of this study was to establish the effects of nicotinamide on the release of labour-promoting mediators in human gestational tissues. Furthermore, we also aimed to characterise the intracellular pathways by which nicotinamide elicits these effects. A well-recognised *ex situ* human tissue explant model [26-28] was used to investigate the effects of increasing concentrations of nicotinamide on pro-labour mediators in human placenta. Tissues were incubated in the presence of lipopolysaccharide (LPS) which is likely to be a factor stimulating the production of cytokines, prostaglandins and oxidative stress in the context of infection-mediated preterm labour. We analysed the effect of nicotinamide on (i) cyclooxygenase (COX)-2, and subsequent prostaglandin (PG)E_2 and $PGF_2\alpha$ production; (ii) pro-inflammatory cytokines (TNF-α and IL-6) and chemokine (IL-8) mRNA expression and release; (iii) 8-isoprostane release and antioxidant mRNA expression; (iv) NF-κB activation and FoxO expression. Knockdown of FoxO3 using siRNA transfection was also performed in primary amnion cells to determine whether nicotinamide elicits its effects via FoxO3.

2. Materials and methods

2.1. Tissue collection and preparation

Human placentae and attached fetal membranes were obtained (with Institutional Research and Ethics Committee approval) from women who delivered healthy, singleton infants at term (>37 weeks' gestation) undergoing elective caesarean section (indications for caesarean section were breech presentation and/or previous caesarean section). Tissues were obtained within 10 min of delivery and dissected fragments were placed in ice-cold DMEM. A placental lobule (cotyledon) was removed from the central region of the placenta. The basal plate and chorionic surface were removed from the cotyledon, and villous tissue was obtained from the middle cross section. Placental tissue was blunt dissected to remove visible connective tissue and calcium deposits. Fetal membranes (choriodecidua and amnion) were prepared by sharp dissection of 2.5-cm^2 squares. Tissue fragments were placed in DMEM at 37°C in a humidified atmosphere of 8% O_2 and 5% CO_2 for 1 h. Explants were blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (100 mg wet weight/well). The explants were incubated, in duplicate, in 2 ml DMEM containing penicillin G (100 U/ml) and streptomycin (100 μg/ml). Explants were incubated, for 24 h, in the presence of 10 μg/ml LPS (to facilitate the production of pro-inflammatory mediators) in the absence or presence of 25 and 50 mM nicotinamide (Sigma, St. Louis, MO, USA). The concentrations used in this study were based on previously published studies for both LPS [26-28] and nicotinamide [13]. After 24 h of incubation, the medium was collected and stored at −80°C until assayed for cytokine, prostaglandin and 8-isoprostane release as detailed below. Tissue was collected and stored at −80°C until assayed for gene expression by quantitative RT-PCR (qRT-PCR), protein expression by Western blotting and NF-κB activation by ELISA.

2.2. Gene silencing of FoxO3 with siRNA in primary amnion cells

Primary amnion epithelial cultures were used to investigate the effect of nicotinamide on siRNA-mediated gene silencing of FoxO3 on pro-inflammatory cytokines, prostaglandins and oxidative stress. Cells were prepared as previously described [33] and incubated in DMEM/F12 enriched with 20% FCS, 10 ng/ml EGF, 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin. Primary amnion cells (Passage 1) at 40–50% confluence in six-well plates were transfected using TransIT-siQUEST reagent according to manufacturer guidelines (Mirus Bio, Madison, WI, USA). Mock transfected cells (transfection reagent only) were used as the vehicle. Cells were transfected with 75 nM FoxO3 siRNA (Ambion, Austin, TX, USA) in DMEM/F12 with 5% FCS for 48 h. The medium was then replaced with DMEM/F12 (with 1% FCS) containing 10 μg/ml LPS with and without 50 mM nicotinamide and the cells were incubated at 37°C for 24 h. Cells and media were collected separately and stored at −80°C.

2.3. Validation of explant cultures and viability

To determine the effect of treatment on cell membrane integrity, the release of the intracellular enzyme lactate dehydrogenase (LDH) into incubation medium was determined as described previously [26-28]. Neither *in vitro* incubation nor experimental treatment significantly affected LDH activity in the incubation medium (data not shown). These data indicate that the concentrations used in this study did not affect cell viability.

2.4. Experimental assays

After 24 h of incubation, the explant incubation medium was collected and the release of IL-6, IL-8 and TNF-α was performed by sandwich ELISA according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The release of PGE_2, $PGF_2\alpha$ and 8-isoprostane into the incubation medium was assayed using a commercially available competitive enzyme immunoassay kit according to the manufacturer's specifications (Kookaburra Kits from Sapphire Bioscience, Redfern, NSW, Australia). All data were corrected for total protein and expressed as either nanograms or picograms per milligram of protein. The protein content of tissue homogenates was determined using BCA protein assay (Pierce, Rockford, IL, USA), using BSA as a reference standard, as previously described [26-28].

2.5. RNA Extraction and quantitative RT-PCR

Total RNA was extracted from approximately 100 mg of tissue and from the primary amnion cell pellet. Extraction was performed using TRI Reagent according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). RNA concentrations were quantified using a spectrophotometer (Smart Spec, Bio-Rad). RNA quality and integrity were determined via the A_{260}/A_{280} ratio. One microgram of RNA was converted to cDNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. The cDNA was diluted 10-fold and 2 μl of this was used to perform RT-PCR using Sensimix Plus SYBR green (Quantace, Alexandria, NSW, Australia) and 100 nM of pre-designed and validated primer (QuantiTect Primer Assays, Qiagen, Germantown, MD, USA). The specificity of the product was assessed from melting curve analysis. RNA without reverse transcriptase during cDNA synthesis as well as PCR reactions using water instead of template showed no amplification. A positive control sample was also used in each run on each plate. Following baseline correction, the fluorescence threshold level was set during the geometric (exponential) phase of PCR amplification to generate the threshold cycle (C_T) value for each amplification curve. Average gene C_T values were normalised to the average actin RNA C_T values of the same cDNA sample. Fold differences were determined using the $2^{-\Delta\Delta C_t}$ method.

2.6. Western blotting

Cytoplasmic protein was extracted as we have previously described [26-28]. Rabbit polyclonal anti-IκBα (MW ~36–38 kDa) and horseradish peroxidase conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Forty micrograms of protein was separated on polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to PVDF. Protein expression was identified by comparison with the mobility of protein standard. Membranes were viewed and analysed using the Chemi-Doc system (Bio-Rad). Quantitative analysis of the relative density of the bands in Western blots was performed using Quantity One 4.2.1 image analysis software (Bio-Rad). Data were corrected for background and expressed as optical density (OD/mm^2).

2.7. Assessment of NF-κB p65 DNA binding activity

NF-κB DNA binding in nuclear protein extracts was assessed using a commercially available NF-κB p65 Transcription Factor ELISA according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Specificity of NF-κB binding was assessed using wild-type and mutated consensus oligonucleotides. A BioRad xMark microplate absorbance spectrophotometer was used to read the sample absorbance, with data expressed as OD at 450 nm.

2.8. Statistical analysis

Statistical analyses were performed using a commercially available statistical software package (Statgraphics Plus version 3.1, Statistical Graphics Corp., Rockville, MD, USA). Homogeneity of data was assessed by Bartlett's test, and when significant, data were logarithmically transformed before further analysis. Data were analysed by one-way ANOVA, and comparisons between groups were performed using Tukey LSD multiple-range tests. Statistical difference was indicated by a P value of less than .05. Data are expressed as mean±S.E.M.

3. Results

3.1. Effect of nicotinamide on pro-inflammatory mediators

To explore the effect of nicotinamide on the inflammatory response in human gestational tissues, we examined the effect of 25 and 50 mM nicotinamide on basal and LPS-stimulated expression of pro-inflammatory cytokines, prostaglandins and oxidative stress in human gestational tissue. These experiments were performed in both placenta villous tissue and fetal membranes. Similar results for placenta and fetal membranes were obtained, but for succinctness, only the data for placenta is shown below.

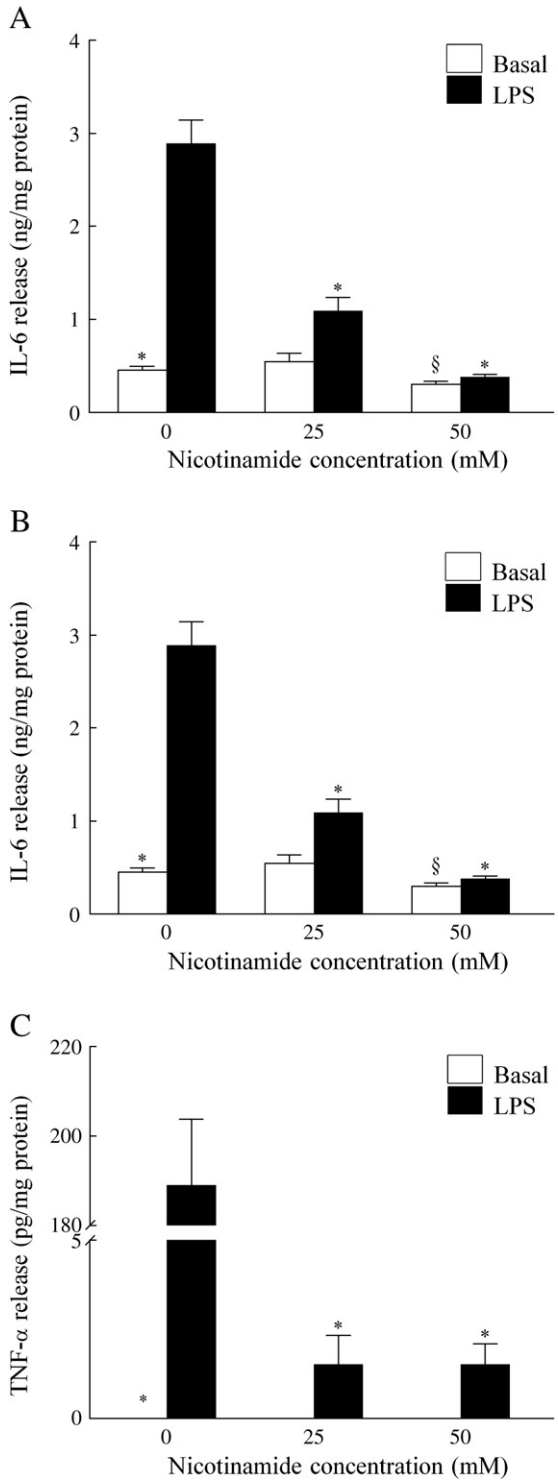

Fig. 1. Effect of nicotinamide on basal and LPS-stimulated (A) IL-6, (B) IL-8 and (C) TNF-α release. Human placenta was incubated for 24 h in the absence or presence of 10 μg/ml LPS with or without 25 and 50 mM nicotinamide. Each bar represents the mean± S.E.M. *P<.05 vs. LPS-stimulated cytokine release; §P<.05 vs. basal cytokine release.

3.1.1. Pro-inflammatory cytokines

LPS (10 μg/ml) significantly stimulated placental cytokine release from placenta (Fig. 1). For IL-6, 25 and 50 mM nicotinamide significantly attenuated LPS-induced release, whereas only the highest concentration of 50 mM inhibited basal release (Fig. 1A). For IL-8, there was no effect of 25 mM nicotinamide on basal and LPS-stimulated release; however, incubation of human placental tissue with 50 mM nicotinamide decreased both basal and LPS-stimulated release of IL-8 (Fig. 1B). TNF-α release was undetectable under basal conditions; however, in the presence of both 25 and 50 mM nicotinamide, there was a significant decrease in LPS-induced release (Fig. 1C).

RT-PCR analyses were performed on RNA isolated from placenta stimulated with 10 μg/ml LPS with and without 50 mM nicotinamide. The effect of nicotinamide treatment on placental gene expression is shown in Table 1. It should be noted that TNF-α was undetectable. LPS induced IL-6 and IL-8 mRNA expression, and co-treatment with nicotinamide significantly suppressed LPS-induced IL-6 and IL-8 gene expression.

3.1.2. Prostaglandins

Placental PGE_2 and $PGF_2\alpha$ release was significantly augmented with 10 μg/ml LPS treatment (Fig. 2). Incubation of tissues with 50 mM nicotinamide significantly diminished the basal release of PGE_2 (Fig. 2A) and $PGF_2\alpha$ (Fig. 2B). Likewise, the addition of both 25 and 50 mM nicotinamide for 24 h suppressed LPS-induced release PGE_2 (Fig. 2A) and $PGF_2\alpha$ (Fig. 2B).

The next aim was to determine whether the effects of nicotinamide on prostaglandin release also occur at the transcriptional level. We thus examined the effect of nicotinamide on the mRNA expression of the time-limiting enzymes involved in prostaglandin formation by COX-2 using qRT-PCR. Significantly augmented COX-2 mRNA expression was detected after 10 μg/ml LPS treatment (Table 1). Co-treatment with 50 mM nicotinamide significantly repressed basal and LPS-stimulated COX-2 mRNA expression.

3.2. Effect of nicotinamide on oxidant status

3.2.1. 8-Isoprostane release

Isoprostanes are prostaglandin-like products derived from free radical-catalysed non-enzymatic oxidation of arachidonic acid [34] and are considered to be an accurate and reliable marker of oxidative stress and endogenous lipid peroxidation [35]; thus in this study the release of 8-isoprostane into incubation medium is used as a marker of oxidative stress, and the effect of nicotinamide on the production of 8-isoprostane from human placenta was examined. Incubation with

Table 1
Effect of 50 mM nicotinamide on placental mRNA expression in the presence of 10 μg/ml LPS

Primer	Basal	LPS	LPS+Nicotinamide
IL-6	0.3±0.1*	1.0±0.0	0.1±0.0*
IL-8	0.1±0.0*	1.0±0.0	0.1±0.1*
TNF-α	0.5±0.1*	1.0±0.0	0.4±0.1*
COX-1	0.9±0.3	1.0±0.0	3.0±0.9*
COX-2	0.3±0.1*	1.0±0.0	0.1±0.0*
FoxO3	1.3±0.4	1.0±0.0	2.6±0.5*

Results (n=3 per group) are illustrated relative to LPS mRNA levels (mean fold change±S.E.M.).

* P<.05 vs. LPS-stimulated mRNA expression (ANOVA).

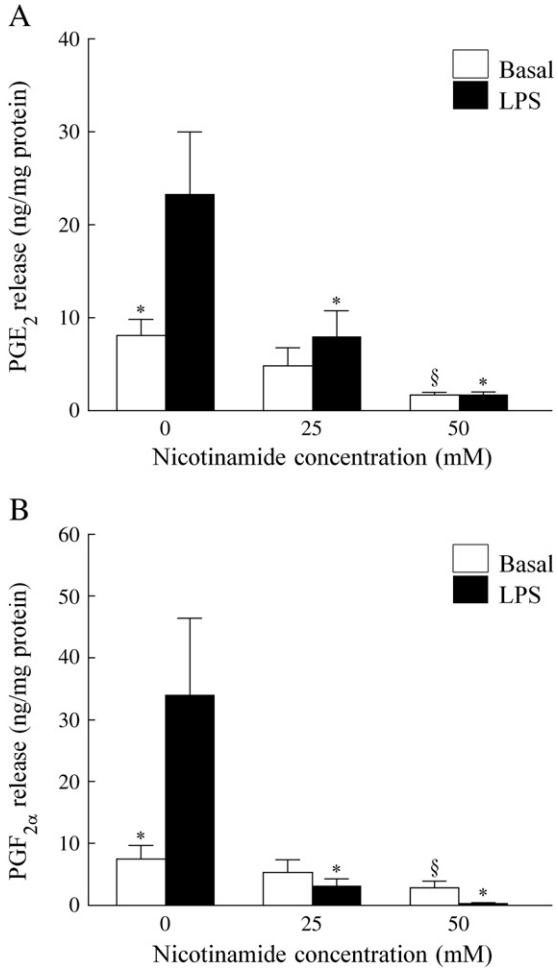

Fig. 2. Effect of nicotinamide on basal and LPS-stimulated (A) PGE_2 and (B) $PGF_2\alpha$ release. Human placenta was incubated for 24 h in the absence or presence of 10 μg/ml LPS with or without 25 and 50 mM nicotinamide. Each bar represents the mean±S.E.M. *P<.05 vs. LPS-stimulated prostaglandin release; §P<.05 vs. basal prostaglandin release.

both 25 and 50 mM nicotinamide significantly reduced basal and LPS-induced 8-isoprostane release (Fig. 3A).

3.2.2. Antioxidant gene expression

qRT-PCR analyses investigating the expression of the following genes — catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GSR) and superoxide dismutase (SOD) — were performed. RNA isolated from placenta was treated with and without nicotinamide in the absence or presence of 10 μg/ml LPS. Basal CAT gene expression was unaffected by nicotinamide treatment (Fig. 3B). LPS treatment significantly reduced CAT mRNA expression (1.1±0.2 vs. 0.5±0.1), and this was significantly increased in the presence of nicotinamide treatment (Fig. 3C). Although there was no effect of LPS treatment on GPx, GSR or SOD gene expression (data not shown), nicotinamide did significantly increase GPx and SOD mRNA expression under both basal (Fig. 3B) and LPS-stimulated (Fig. 3C) conditions. There was, however, no effect of nicotinamide treatment on GSR mRNA expression.

3.3. Mechanisms by which the anti-inflammatory actions of nicotinamide are elicited

3.3.1. Nuclear factor-κB

It has recently been shown that nicotinamide inhibits pro-inflammatory cytokines via NF-κB [18]. Therefore, we aimed to

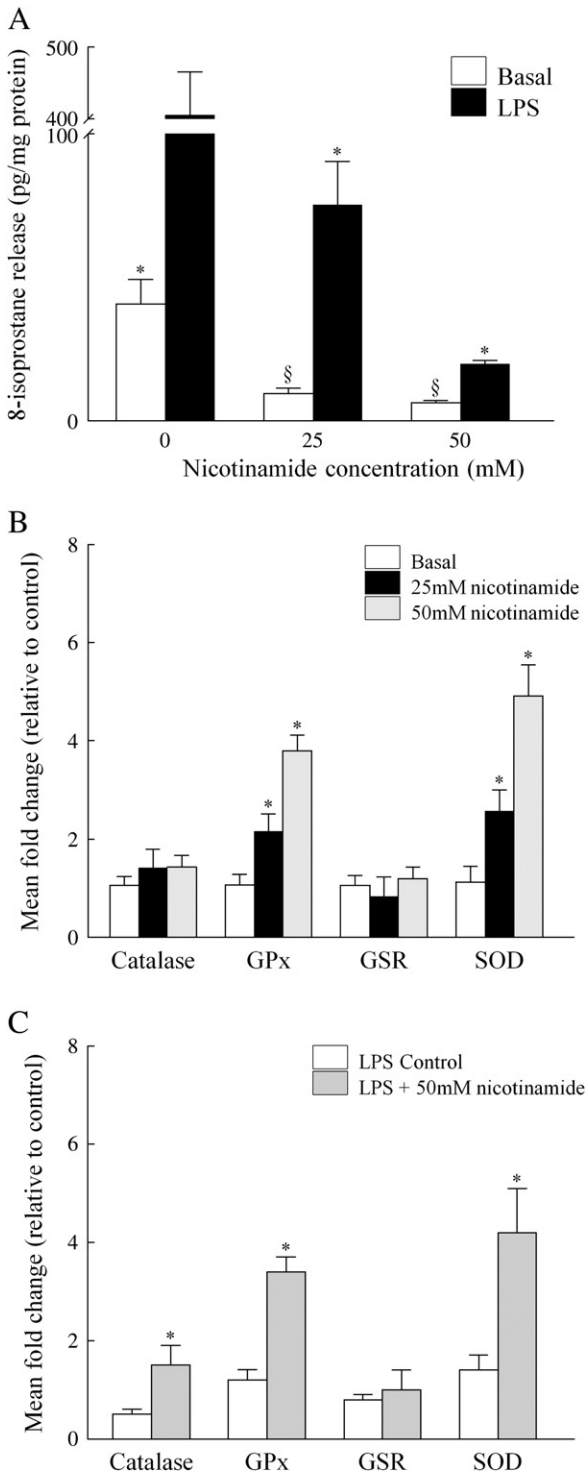

Fig. 3. Nicotinamide regulates oxidative stress in human placenta. (A) Effect of nicotinamide on basal and LPS-stimulated 8-isoprostane release. Human placenta was incubated for 24 h in the absence or presence of 10 μg/ml LPS with or without 25 and 50 mM nicotinamide. Each bar represents the mean±S.E.M. *P<.05 vs. LPS-stimulated 8-isoprostane release; §P<.05 vs. basal 8-isoprostane release. (B) Effect of nicotinamide on basal antioxidant gene expression. Human placenta was incubated 24 h in the absence or presence of 25 and 50 mM nicotinamide. Each bar represents the mean fold change±S.E.M. *P<.05 vs. basal antioxidant gene expression. (C) Effect of nicotinamide on antioxidant gene expression in the presence of LPS. Human placenta was incubated for 24 h in the absence or presence of 10 μg/ml LPS with or without 50 mM nicotinamide. Each bar represents the mean fold change±S.E.M. *P<.05 vs. LPS-induced antioxidant gene expression.

determine whether the observed anti-inflammatory actions of nicotinamide in human gestational tissues are also due to inhibiting NF-κB activation. The binding ability of NF-κB p65 to DNA consensus sequences was also measured using a commercially available kit. An NF-κB wild-type consensus oligonucleotide was used to monitor the specificity of the assay. The wild-type oligonucleotide, by competing for NF-κB binding to the probe immobilised on the plate, acted as an effective competitor for NF-κB p65 binding (data not shown). Nicotinamide had no effect on basal or LPS-stimulated NF-κB p65 DNA binding activity (Fig. 4A). Likewise, nicotinamide had no effect on LPS-induced IκB-α protein degradation (Fig. 4B) in human placenta. Similar results were obtained for fetal membranes (data not shown).

3.3.2. FoxO3

In non-gestational tissues, the actions of nicotinamide are in part due to activation of FoxO proteins [9,10,25]. In support of this, we show that nicotinamide activates FoxO3 mRNA expression in human placenta (Table 1). The next aim was then to determine whether the observed anti-inflammatory actions of nicotinamide are mediated by FoxO3. To elucidate the cellular mechanism underlying the protective effects of nicotinamide, the knockdown of FoxO3 mRNA (Fig. 5A) was achieved by RNA interference in primary amnion cells. Specifically, when compared to mock transfected cells, incubation of FoxO3-deficient primary amnion cells with nicotinamide (in the presence of LPS) augmented IL-8 and COX-2 gene expression (Fig. 5B) and significantly increased the release of IL-8, PGE_2, $PGF_2\alpha$ and 8-isoprostane (Fig. 5C).

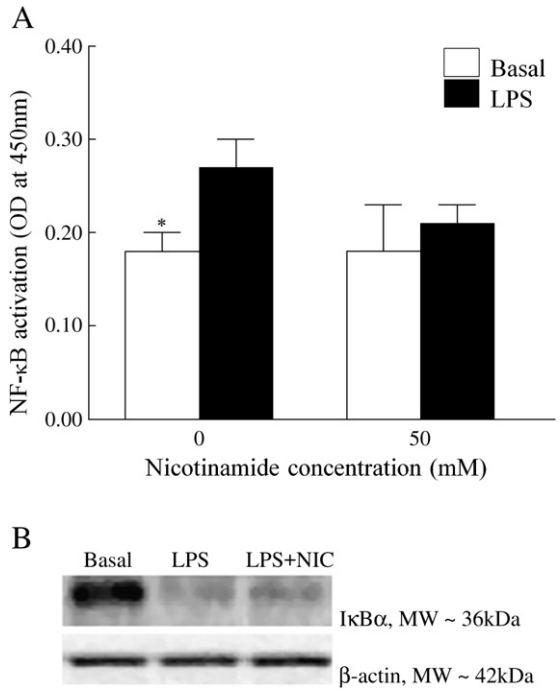

Fig. 4. (A) Effect of nicotinamide on basal and LPS-stimulated NF-κB p65 activity. Human placenta was incubated for 24 h in the absence or presence of 10 μg/ml LPS with or without 50 mM nicotinamide. Each bar represents the mean OD±S.E.M. *$P<.05$ vs. LPS-stimulated NF-κB p65 activity. (B) Western blot demonstrating the effect of nicotinamide on IκBα protein expression in human placenta. Human placenta was incubated for 24 h in the absence or presence of 10 μg/ml LPS with and without 50 mM nicotinamide (NIC). Data are representative of $n=4$ experiments.

4. Discussion

The data presented in this study demonstrate that the vitamin B_3 derivative nicotinamide exerts anti-inflammatory and antioxidative actions in human gestational tissues. Specifically, we show that nicotinamide attenuates basal and/or LPS-induced increases in cytokines' gene expression and release, and COX-2 mRNA expression and resultant prostaglandin release. Additionally, in the presence of nicotinamide, basal and LPS-induced 8-isoprostane release is suppressed, whereas antioxidant gene expression is increased. We demonstrate that the anti-inflammatory and antioxidant actions of nicotinamide are elicited via FoxO3.

In this study, we have shown that nicotinamide significantly inhibits basal and LPS-induced secretion and mRNA expression of pro-inflammatory cytokines from human gestational tissues. This has important implications as the coordinated interplay of anti- and pro-inflammatory cytokines and chemokines regulates many of the processes of human labour and delivery, both at term and at preterm [7]. Pro-inflammatory cytokines increase with advancing gestation, advancing labour and in infection-associated premature deliveries [4,7]. These pro-inflammatory cytokines can then induce (i) further cytokine release through a positive feed forward mechanism; (ii) the expression of adhesion molecules; (iii) phospholipid metabolising enzymes and their products; and (iv) ECM remodelling enzymes. This provides a network that facilitates remodelling of gestational tissues to facilitate delivery. Our finding that nicotinamide suppresses pro-inflammatory cytokines is in agreement with other studies. Studies in non-gestational tissues demonstrate that nicotinamide can regulate cellular inflammation. For example, nicotinamide blocks pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNF-α [11,13,15,16,18-20], and transforming growth factor (TGF)-β2 and macrophage chemotactic protein-1 [17]. Yet, the role of nicotinamide during inflammation is not entirely clear, since some investigations that examined the ability of oral nicotinamide administration to reduce cytokine production following endotoxin challenge in healthy volunteers did not demonstrate a significant effect upon serum cytokine levels [36].

PGE_2 and $PGF_2\alpha$, formed from arachidonic acid through activity of the COX enzyme complex, are produced by maternal and fetal tissues during parturition, and changes in the concentrations of prostaglandins correlate with the physiological changes that occur during labour [37]. Prostaglandins play an important role in human parturition, stimulating uterine contractions and cervical ripening during human labour [37], and administration of prostaglandin synthase inhibitors suppresses uterine activity and prolongs the length of pregnancy [38]. In this study, treatment with nicotinamide inhibited both basal and LPS-induced release of PGE_2 and $PGF_2\alpha$, and the gene expression of COX-2. Similarly, nicotinamide decreases LPS-stimulated PGE_2 in macrophages [13].

Oxidative stress is a biochemical condition that is characterised by the imbalance between the presence of relatively high levels of toxic reactive species, principally consisting of ROS, and the body's scavenging ability (antioxidants) [39]. Increased oxidative stress has been implicated in the processes of human preterm and term labour [5,6]. In keeping with this, ROS is activated by microorganism infection, cigarette smoking and expression of cytokines during pregnancy. In this study, we revealed that nicotinamide attenuated basal and LPS-stimulated 8-isoprostane release; this was associated with an increase in the antioxidant enzymes SOD and GPx. Consistent with the data presented in this study, nicotinamide has been shown *in vitro* to reduce the generation of ROS [13,24] and increase the expression of a number of antioxidant enzymes [21-23].

There is much evidence that NF-κB regulates pro-inflammatory cytokines, prostaglandins and ECM remodelling enzymes in human

gestational tissues, suggesting the role of NF-κB in the processes of human labour and delivery [26-32]. A recent study has shown that nicotinamide modulates NF-κB activation in mortalised HaCaT cells and primary keratinocytes stimulated by *Propionibacterium acnes* [18]. In this investigation, however, nicotinamide did not attenuate LPS-induced IκB-α degradation or NF-κB activation, suggesting that the anti-inflammatory actions of nicotinamide in human gestational tissues are not elicited via NF-κB.

Other studies have reported that nicotinamide modulates the expression of FoxO proteins [9,10,25]. FoxO proteins are involved in multiple signalling pathways and play important roles in a number of physiological processes including cellular differentiation, tumour suppression, metabolism, cell-cycle arrest, cell death and protection from oxidative stress [40-45]. In this study, we showed that nicotinamide treatment induced FoxO3 gene expression. A role for both FoxO3 in the anti-inflammatory and antioxidative effects of nicotinamide is demonstrated by the findings that knockdown of this transcription factors attenuated the inhibitory effect of nicotinamide on the LPS-induced up-regulation of pro-labour mediators. Our data is in keeping with studies demonstrating an important role for FoxO3 in the regulation of inflammation *in vitro* and *in vivo*. For example, mice deficient in FoxO3 develop spontaneous, multisystemic inflammatory syndrome, which is associated with increased cytokine production [46]. Furthermore, overexpression of FoxO3 in T cells inhibits cytokine expression [46]. Bacterial infection inactivates FoxO3 leading to increased cytokine expression in intestinal epithelia [47], and in HT-29 cells, TNF-α inactivation of FoxO3 increases IL-8 [48]. Knockdown of FoxO3 also leads to an up-regulation of oxidative stress in human dermal fibroblasts [49]. Likewise, *FoxO3* deficiency increases eNOS expression [50], an enzyme that is increased during labour [51].

In summary, nicotinamide, via activation of FoxO3, can abrogate the inflammatory processes of human labour in human gestational tissues induced by bacterial endotoxin. These observed anti-inflammatory and antioxidative properties exhibited by nicotinamide in human gestational tissues would thus be expected to favour the maintenance of pregnancy. This has important implications as maternal cervical and intrauterine infection and inflammation may have a primary causative role in many cases of preterm labour [52]. Thus, B_3 vitamins and their derivatives, or targeting medicinal agents towards FoxO3, may potentially be of benefit to preterm parturition. Indeed, the clinical importance of activating FoxO3 and its downstream genes in various anticancer therapeutics has been reported by many [53].

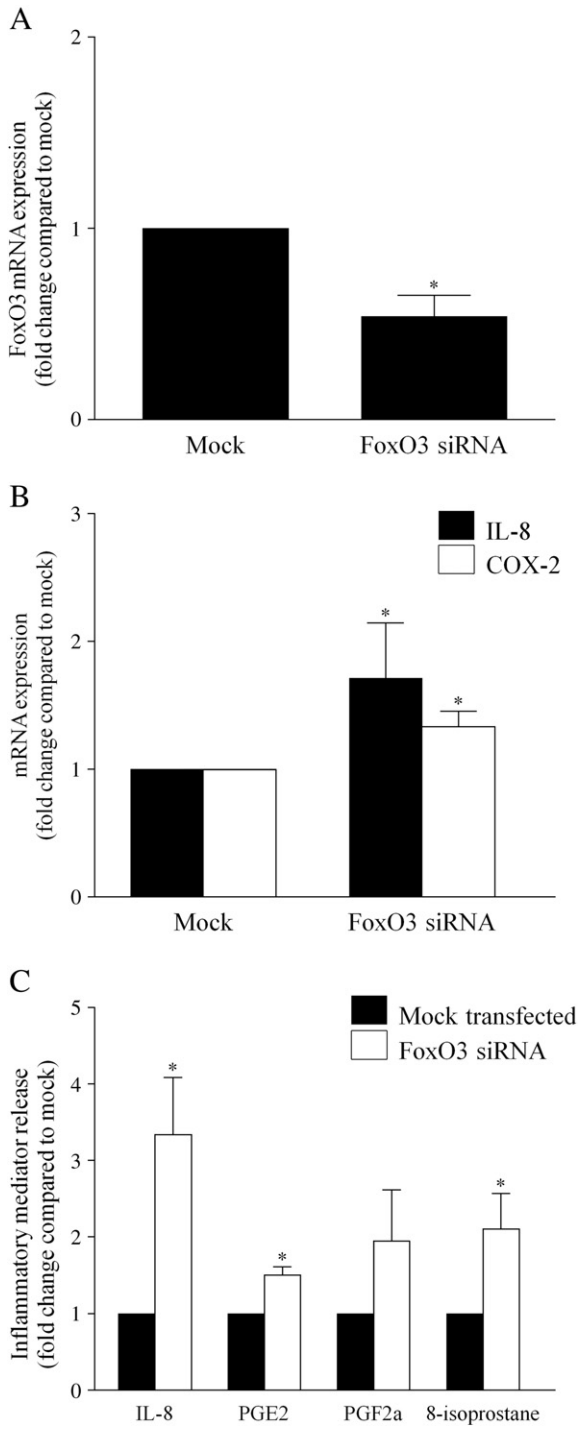

Fig. 5. FoxO3 is involved in the anti-inflammatory effects of nicotinamide. (A) Efficiency of knockdown of FoxO3 mRNA in primary amnion cells using siRNA. After 48 h of transfection with FoxO3 siRNA, human primary amnion cells were incubated with 50 mM nicotinamide for 24 h in the presence of 10 μg/ml LPS (n=3 independent experiments). RNA was extracted, converted to cDNA and the expression was analysed by qRT-PCR as detailed in the Materials and methods section. 18S mRNA expression was used for the normalisation of the data. Gene expression is displayed as mean fold change (compared to mock)±S.E.M. (n=3 per group). *P<.05 vs. mock mRNA expression. (B) Effect of nicotinamide on IL-8 and COX-2 mRNA expression. After 48 h of transfection with FoxO3 siRNA, human primary amnion cells were incubated with 50 mM nicotinamide for an additional 24 h in the absence or presence of 10 μg/ml LPS. Cells were collected, and RNA was extracted, converted to cDNA and the expression was analysed by qRT-PCR as detailed in the Materials and methods section. 18S mRNA expression was used for the normalisation of the data. Gene expression is displayed as mean fold change (compared to LPS plus nicotinamide mock transfected)±S.E.M. (n=3 per group). *P<.05 vs. LPS plus nicotinamide mock transfected gene expression. (C) Effect of nicotinamide on pro-labour mediator release. After 48 h of transfection with FoxO3 siRNA, human primary amnion cells were incubated with 50 mM nicotinamide for an additional 24 h in the absence or presence of 10 μg/ml LPS. Media was collected and assayed for the release of cytokine, prostaglandins and 8-isoprostane enzyme activity as detailed in the Materials and methods section. Each bar represents the mean fold change (compared to LPS plus nicotinamide mock transfected)±S.E.M. *P<.05 vs. LPS plus nicotinamide mock transfected release.

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