

Maternal metabolic stress may affect oviduct gatekeeper function

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Abstract

We hypothesized that elevated non-esterified fatty acids (NEFA) modify *in vitro* bovine oviduct epithelial cell (BOEC) metabolism and barrier function. Hereto, BOECs were studied in a polarized system with 24-h treatments at Day 9: (1) control (0 μ M NEFA + 0% EtOH), (2) solvent control (0 μ M NEFA + 0.45% EtOH), (3) basal NEFA (720 μ M NEFA + 0.45% EtOH in the basal compartment) and (4) apical NEFA (720 μ M NEFA + 0.45% EtOH in the apical compartment). FITC-albumin was used for monolayer permeability assessment and related to transepithelial electric resistance (TER). Fatty acid (FA), glucose, lactate and pyruvate concentrations were measured in spent medium. Intracellular lipid droplets (LD) and FA uptake were studied using Bodipy 493/503 and immunolabelling of FA transporters (FAT/CD36, FABP3 and CAV1). BOEC-mRNA was retrieved for qRT-PCR. Results revealed that apical NEFA reduced relative TER increase (46.85%) during treatment and increased FITC-albumin flux (27.59%) compared to other treatments. In basal NEFA, FAs were transferred to the apical compartment as free FAs: mostly palmitic and oleic acid increased respectively 56.0 and 33.5% of initial FA concentrations. Apical NEFA allowed no FA transfer, but induced LD accumulation and upregulated FA transporter expression (\uparrow CD36, \uparrow FABP3 and \uparrow CAV1). Gene expression in apical NEFA indicated increased anti-apoptotic (\uparrow BCL2) and anti-oxidative (\uparrow SOD1) capacity, upregulated lipid metabolism (\uparrow CPT1, \uparrow ACSL1 and \downarrow ACACA) and FA uptake (\uparrow CAV1). All treatments had similar carbohydrate metabolism and oviduct function-specific gene expression (OVGP1, ESR1 and FOXJ1). Overall, elevated NEFAs affected BOEC metabolism and barrier function differently depending on NEFA exposure side. Data substantiate the concept of the oviduct as a gatekeeper that may actively alter early embryonic developmental conditions.

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Introduction

In dairy cattle, extensive genetic selection to promote milk yield has led to a drastic increase in energetic demands and reduced fertility (Leroy *et al.* 2008a,b). To support increased milk production, dairy cow metabolism shifts to prioritize lactation, causing metabolic stress, which can be manifested through increased lipolysis and elevated serum concentrations of non-esterified fatty acids (NEFAs) (Leroy *et al.* 2005). Similar observations have been described in women in whom metabolic stress, associated with e.g. obesity and type II diabetes, is linked with lipolytic disorders (Lash & Armstrong 2009).

Elevated serum NEFAs are reflected in the ovarian follicular fluid (Leroy *et al.* 2004, Leroy *et al.* 2005,

Robker *et al.* 2009) and are recognized as important factors affecting fertility. As such, NEFAs have direct detrimental effects on murine folliculogenesis (Valckx *et al.* 2014), bovine oocyte nuclear maturation and developmental capacity (Jorritsma *et al.* 2004, Leroy *et al.* 2005, Aardema *et al.* 2011, Van Hoeck *et al.* 2011) and the quality of the resulting embryo (Van Hoeck *et al.* 2011). In women and mice, oocyte quality has also been related to metabolic alterations in follicular fluid (Valckx *et al.* 2012, 2015) with potentially lasting adverse effects in the offspring (Jungheim *et al.* 2011).

In addition, it has been demonstrated that elevated NEFAs can affect *in vitro* bovine oviduct epithelial cell (BOEC) physiology (Jordaens *et al.* 2015). Elevated NEFAs hampered BOEC physiology by reducing cell proliferation, cell migration capacity, cell functionality

and monolayer integrity, in a cell polarity-dependent manner. However, insights in the pathways associated to these observations and in cellular responses arising from NEFA exposure are currently lacking. Furthermore, it's important to learn 'how', 'whether' and 'to which extent' intracellular fatty acid (FA) uptake and transepithelial transfer of these FAs can occur. Recent *in vivo* experiments indeed indicated that the conditions in the reproductive tract define its ability to sustain early embryo development (Rizos *et al.* 2010, Maillo *et al.* 2012, Matoba *et al.* 2012). As such, the oviductal environment in metabolically stressed lactating dairy cattle was less supportive for blastocyst formation compared to heifers (Rizos *et al.* 2010) and to non-lactating cows (Maillo *et al.* 2012). *In vitro* reports suggest this may be due to direct environmental effects of elevated NEFAs, as NEFA exposure during bovine embryo culture jeopardized embryo quality through reduced blastocyst formation and cell number, with a concomitant rise in apoptosis (Van Hoeck *et al.* 2013) and internalization of FAs (Listenberger *et al.* 2003, Leroy *et al.* 2010). In mice, similar observations have been made as exposure of murine embryos to pathological NEFA concentrations during *in vitro* culture, induced effects on embryo metabolism and growth (Jungheim *et al.* 2011). However, whether or not elevated serum NEFAs can be transferred across the oviduct epithelial lining and are actually reflected in the oviductal lumen, where they may contribute to suboptimal embryo growth conditions, remains to be elucidated.

It is furthermore unknown whether elevated NEFA concentrations may influence oviduct-specific characteristics such as permeability. Earlier Roche and coworkers (Roche *et al.* 2001) reported that FAs altered *in vitro* Caco-2 monolayer confluency by affecting transepithelial electric resistance (TER) and expression of tight junctions. In oviductal cells, a reduced TER and cell migration capacity were observed in the presence of elevated NEFAs (Jordaens *et al.* 2015), but mechanistic insights are currently lacking. Affecting oviduct epithelial permeability and thereby altering the oviduct gatekeeper function would reflect in the overall composition of the oviduct microenvironment as different molecules may be filtered from the serum to the oviductal lumen (Leese *et al.* 2007). NEFAs may therefore also indirectly affect early embryo development.

Studies expanding on the consequences of elevated NEFAs on oviduct cell function and micro-environment are scarce. Possibly, as *in vivo* studies remain challenging to perform due to specialist equipment and techniques and difficult to interpret considering the complexity of the whole organism (Velazquez *et al.* 2010). Hereto, an *in vitro*-polarized cell culture (PCC) system with hanging inserts (Miessen *et al.* 2011, Tahir *et al.* 2011) may provide a valid alternative as it promotes the preservation of both

morphology and biology of native oviduct epithelium (Fotheringham *et al.* 2011) while focusing on immediate cellular responses of oviduct epithelial cells exclusively. It is therefore considered as a valuable tool to acquire primary mechanistic insights in the direct effects of NEFAs on BOEC physiology. In particular, BOEC metabolism and barrier function, oviduct-specific functions such as oviduct specific glycoprotein secretion, anti-oxidative and anti-apoptotic characteristics and cellular FA transfer or uptake are of interest, as they may influence early embryo development.

Therefore, in the present study, we hypothesized that elevated NEFA concentrations can affect BOEC physiology by altering BOEC metabolism and barrier function. Hereto, we aimed to obtain a more profound understanding in the direct effects of elevated NEFAs on BOEC physiology and gatekeeper features in a PCC by observing (1) BOEC monolayer integrity and permeability, (2) FA transfer across the monolayers, (3) intracellular lipid accumulation, (4) BOEC FA transporters, (5) BOEC energy metabolism and (6) mRNA expression of genes related to BOEC viability, oxidative stress, BOEC-specific functions and both carbohydrate and lipid metabolism. This research may ultimately further elucidate the direct effects of NEFAs on the oviductal micro-environment, affecting pre-implantation embryo development. This may contribute to the complex pathogenesis of infertility associated with lipolytic metabolic disorders.

Materials and methods

All chemicals were purchased from Thermo Fisher Scientific, unless stated otherwise.

Primary BOEC culture: isolation and culture in a polarized cell culture (PCC) system

BOECs were isolated and cultured as described previously (Jordaens *et al.* 2015). Briefly, in each replicate, 4 bovine oviducts from cows in the early luteal phase (days 3–5 of the estrous cycle) and ipsilateral to the ovulation site were obtained from a local slaughterhouse. As the pre-implantation embryo interacts with both ampulla and isthmus, BOECs from whole oviducts were mechanically isolated within 3 h after slaughter. BOEC number and viability were determined, using trypan blue exclusion and a hemocytometer and seeded at a density of 1×10^6 cells/mL in a polarized cell culture (PCC) system with hanging inserts (Corning, Snapwell, 6-well). Each compartment contained 2 mL culture medium, based on DMEM/F12 (containing 0.75% w/v BSA (essentially FA free; Sigma-Aldrich), 5% v/v serum (2.5% v/v Fetal Bovine serum, Greiner Bio-One, Frickenhausen, Germany; and 2.5% v/v Newborn Calf Serum, Sigma-Aldrich, St-Louis, MO, USA), 2.5% v/v penicillin/streptomycin and 2% v/v amphotericin B) and was renewed initially after 24 h, subsequently every 48 h.

Preparation of the treatments

The types and concentrations of free FAs used are based on the *in vivo* concentrations found in the serum of high-yielding dairy cows in negative energy balance (NEB) (Leroy *et al.* 2005). To mimic the FA profile during NEB, NEFA concentrations of 720 μM (i.e. 230 μM palmitic acid (PA)+280 μM stearic acid (SA)+210 μM oleic acid (OA)) were implemented as a pathological condition and prepared as described by Van Hoeck and coworkers (Van Hoeck *et al.* 2011). Solubility of lipophilic NEFAs into hydrophilic culture was spectrophotometrically confirmed prior to use.

Experimental design

BOECs were maintained in hanging inserts and supported by medium replenishments of both compartments every 48 h until they reached confluency, as confirmed by transepithelial electrical resistance (TER) using an Avometer (Millicell-ERS, Millipore). Monolayer formation was defined confluent when the TER recordings exceeded 700 Ωcm^2 (Chen *et al.* 2015) at Day 9. Ultimately at Day 9, pre-exposure medium samples were collected after which 4 treatments were established: (1) control: 0 μM NEFA in both compartments, (2) solvent control: 0 μM NEFA+0.45% v/v EtOH in both compartments, (3) basal NEFA: 720 μM NEFA+0.45% v/v EtOH in the basal compartment and (4) apical NEFA: 720 μM NEFA+0.45% v/v EtOH in the apical compartment. Preparations of NEFA were added to the monolayers at Day 9 for 24 h as depicted in Fig. 1. After 24 h (Day 10), outcome parameters were assessed, spent medium from both compartments in all wells was sampled and BOECs were either collected using EDTA–trypsin for mRNA extraction or fixed in 4% paraformaldehyde for

immunofluorescent staining. Per outcome parameter, samples from a total of 16 animals were used and analyzed as four pools of four.

Outcome parameters

BOEC integrity and monolayer permeability

TER measurements were recorded, both before (Day 9) and after NEFA exposure (Day 10) to observe monolayer confluence and integrity. Hereto, a Millicell-ERS (Millipore) was used according to the manufacturer's instructions. Monolayers were considered confluent when TER values ranged between 700 and 1100 Ωcm^2 (Chen *et al.* 2015). Data were expressed as relative TER increase over the 24-h treatment period. At Day 10 and immediately after NEFA exposure, monolayer permeability was determined by measuring macromolecular transport of 66 kDa FITC-labeled albumin across the monolayers, as described by Chang and coworkers (Chang *et al.* 2000) in endothelial cells with some modifications to suit our design, objective and cell type. Briefly, in 4 repeats (2 inserts per flux direction within each treatment group and per replicate, total number of inserts $n=54$), FITC-albumin (15 μM) was dissolved in HBSS without phenol red and added to either the apical or the basal chamber (each 2 wells per treatment per replicate) to observe albumin flux in either direction. Unseeded inserts were used as a positive control to exclude effects due to the membrane properties. After 3 h, medium in each compartment was mixed by pipetting and 20 μL samples were submitted for FITC measurement at 490 nm excitation/530 nm emission using a Tecan microplate reader, Infinite 200 Pro (Tecan Trading AG, Switzerland). Both the supplemented and the non-supplemented compartments were sampled to retrospectively

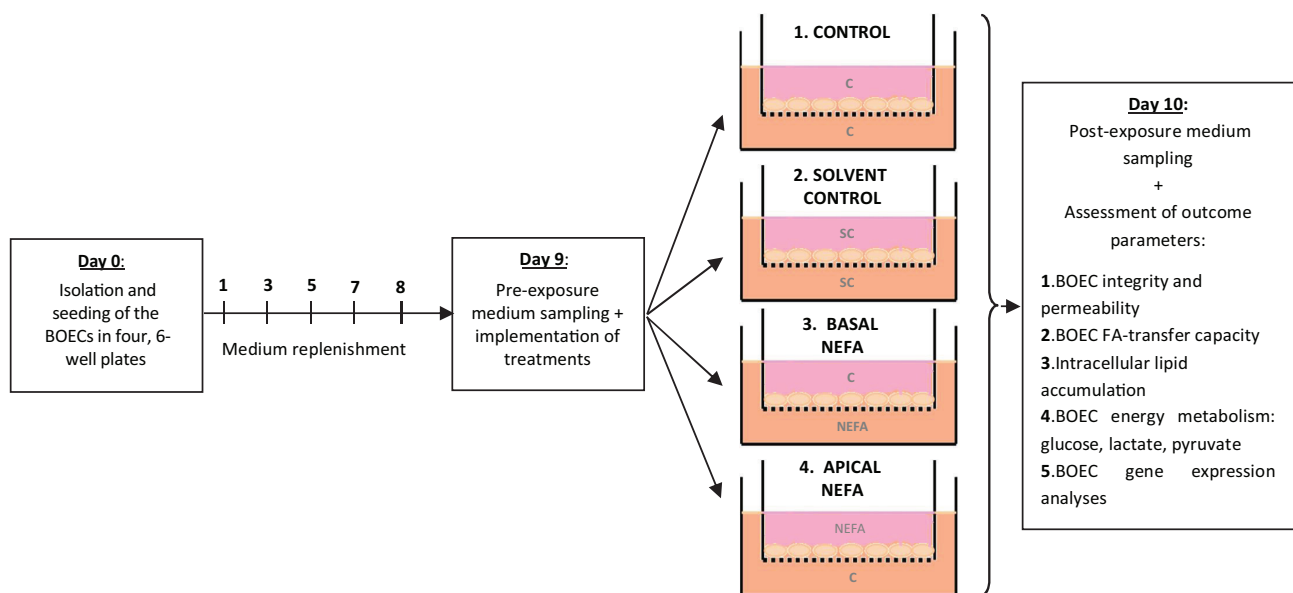


Figure 1 Experimental design to study the effects of NEFAs in a polarized cell culture system according to different exposure directions: C, control medium containing 0 μM NEFA; SC, solvent control medium containing 0 μM NEFA+0.45% EtOH; NEFA, 720 μM NEFA with 230 μM PA+280 μM SA+210 μM OA. At Days 9 (pre-exposure samples) and 10 (post-exposure samples), spent media were collected. At Day 10, other outcome parameters in BOECs were assessed.

correlate the decrease in fluorescence from the supplemented compartment to the increase in fluorescence in the non-supplemented compartment. Standard curves ranged from 0 to 2 μM , however, to match the FITC-albumin concentrations in the supplemented compartment, a 10 \times dilution was required. R^2 values of >0.99 and CV <10% were considered valid.

BOEC fatty acid transfer capacity

Spent medium from both NEFA-supplemented and their opposite compartments were spectrophotometrically analyzed for total FA concentrations, gas chromatographically for individual FA concentrations and for FA profiles per FA fraction (free or esterified) in 4 repeats.

Total FA concentrations: Total FA concentrations were measured at the 'Algemeen Medisch Labo' (AML, Antwerp, Belgium), using commercial photometric assays, RX Daytona (Randox Laboratories) in 4 replicates with 3 observations per treatment. Measurements were carried out according to manufacturer's instructions. The intra- and inter-assay coefficients of variation for all analyses were <5%.

FA profiles per FA fraction (free or esterified): FAs in spent medium (in 4 replicates using a pool of 2 inserts per treatment) were extracted as described by Löfgren and coworkers (Löfgren *et al.* 2012), with heneicosanoic acid (5 μg) and triheptadecanoin (5 μg) as internal standards. The FA extract was divided in three aliquots for the determination of (i) total FAs, (ii) FAs in triacylglycerols, cholesteryl esters and glycerophospholipids (esterified) and (iii) non-esterified fatty acids (free).

Total FAs were methylated by a consecutive base-catalyzed and an acid-catalyzed step (Vlaeminck *et al.* 2014). Esterified FAs (in triacylglycerols, cholesteryl esters and glycerophospholipids) were methylated using only the base-catalyzed step. For separation of the free FAs, the NEFA-containing hexane layer was methylated using an acid-catalyzed step. Fatty acid methyl esters were subsequently extracted with hexane.

Composition analysis of FA-methyl esters was carried out by gas chromatography (HP7890A, Agilent Technologies) with a split-splitless injector and flame ionization detector using a SP-2560 column (75 m \times 0.18 mm, i.d. \times 0.14 μm thickness, Supelco Analytical, Bellefonte, USA). The carrier gas was hydrogen (flow rate: 1 mL/min) with splitless injection (t° : 50°C for 2.5 min, 175°C for 13 min and 215°C for 25 min). Inlet and detector temperatures were 250 and 255°C respectively. Peaks were identified based on retention time comparisons with a mixture of FAME standards (GLC463, Nu-Check-Prep., Inc., Elysian, MN, USA). Quantification of FA-methyl esters was based on the area of the internal standard and on the conversion of peak areas to the weight of FAs by a theoretical response factor for each FA (Ackman & Sipos 1964, Wolff *et al.* 1995).

Intracellular lipid accumulation

In three replicates, monolayers from 3 inserts per treatment were fixed at Day 10 of culture (and after NEFA exposure according to Fig. 1) in 4% phosphate buffered paraformaldehyde for 10 min. BOECs were washed twice with

DPBS and permeabilized with saponin (0.1% w/v) (Carl Roth GmbH&Co, Karlsruhe, Germany). Nuclei were stained with 5 $\mu\text{g}/\text{mL}$ DAPI (Molecular Probes) for 5 min and subsequently washed with DPBS. Neutral lipids were stained with BODIPY 493/503 (Molecular Probes) (20 $\mu\text{g}/\text{mL}$) in DPBS for 1 h, according to a modified protocol of Van Hoeck and coworkers (Van Hoeck *et al.* 2013). After staining the insert membranes and monolayers were removed from the insert housing and mounted on a microscope slides with Citifluor (VWR, Haasrode, Belgium). High-resolution images were obtained using Nikon Eclipse Ti-E inverted microscope, attached to a microlens-enhanced dual-spinning disk confocal system (UltraVIEW VoX; PerkinElmer) equipped with 405 and 488 nm diode lasers for the excitation of blue and green fluorophores respectively. For each monolayer, 10 random z-stack of 20 μm with each 1 μm intervals were made starting at the level of the insert membrane. In extended focus images, neutral lipid accumulation was compared qualitatively among treatments.

BOEC fatty acid transporters

At Day 10 of culture (and after NEFA exposure according to Fig. 1), 1 BOEC monolayer per treatment was fixed in 4% phosphate buffered paraformaldehyde for 10 min in 3 replicates. Monolayers were submitted to immunofluorescent staining, using polyclonal anti-FABP3 rabbit anti-bovine antibodies (MyBiosource), polyclonal anti-CD36 rabbit anti-bovine antibodies (Thermo Fisher Scientific) or polyclonal anti-CAV1 rabbit anti-bovine antibodies (Cell Signaling Technology). FITC-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific) was used as secondary antibody according to manufacturer's instructions. Protocols were tested for non-specific primary and secondary antibody binding, and bis benzimide (Hoechst no 33342; Sigma-Aldrich) was used as nuclear stain. After staining, the insert membranes and monolayers were removed from the insert housing and mounted on a microscope slides with Citifluor (VWR). High-resolution images were obtained using Nikon Eclipse Ti-E inverted microscope (vide supra '3. Intracellular lipid accumulation'). For each monolayer, full thickness z-stacks with 0.5 μm intervals were randomly made to localize the BOEC FA transporter expression. To quantify the BOEC FA transporter expression, 10 random single z-plane images per monolayer were made. Laser settings for the 405 nm laser line were used to focus all nuclei in each plane, whereas 488 nm laser settings were fixed for each transporter type. In each image, total green fluorescence and number of nuclei were measured using Volocity imaging software, version 6.3.1 (PerkinElmer). The level of FA transporter expression is presented as the mean amount of green fluorescent pixels counted per nucleus.

BOEC energy metabolism: glucose, lactate and pyruvate concentrations

Medium sampling was performed pairwise as repeated measures at Days 9 and 10: pre-exposure medium (routine BOEC, DMEM/F12-based culture medium) was added at Day 8 and sampled at Day 9 after 24-h incubation (4 replicates

with 3 observations per treatment). Post-exposure medium, containing the different treatments, was subsequently added at Day 9 and sampled 24 h later at Day 10. Both pre- and post-exposure media were prepared from the same batch to assure all composing nutrients were identical. Immediately after collection, all medium samples were centrifuged at 1250 g (5 min, room temperature) to avoid cellular contamination and possible confounding of the results by ongoing cellular activities in the medium. Subsequently, samples were snap frozen at -196°C in liquid nitrogen and stored at -80°C until further analysis. All analyses were performed within 3 months after sample collection. Lactate production and glucose and pyruvate consumption ($n=96$: 4 replicates, 4 treatments, 3 wells per treatment with both an apical and a basolateral compartment) were determined by an ultrafluorometric assay of spent medium as described by Gardner and Leese (1990), with modifications by Guerif and coworkers (Guerif *et al.* 2013) using a Tecan microplate reader, Infinite 200 Pro (Tecan Trading AG, Switzerland). Blank medium aliquots (with no cellular contact) were collected to calculate the consumption/production, and data were expressed as nmol/well/h. As differences in consumption or production data in the pre-exposure samples can only be due to cell number, these values were used to normalize post-exposure data. Data were expressed as a relative increase over the 24-h exposure period.

BOEC gene expression analyses

Gene expression analyses were performed using two BOEC monolayers per treatment in 4 replicates. The extraction of total RNA from cells was carried out using TRIzol reagent according to manufacturer's instructions. The isolated RNA was suspended in 1 mL of isopropanol for at least 20 min. Subsequently, the isopropanol was vaporized in a vacuum chamber, and the RNA pellet was washed in 70% ethanol. Subsequently, mRNA was selected using the Dynabeads mRNA DIRECT Micro Kit (Ambion, Thermo Fisher Scientific) according to manufacturer's instructions with minor modifications (Bermejo-Álvarez *et al.* 2008). To eliminate potential contamination with genomic DNA, all samples were incubated with DNase, at 37°C for 30 min and at 90°C for 5 min (RQ1 RNase-Free DNase, Promega). RNA concentration was quantified at a wavelength of 260 nm, and purity was assessed by the 260/280 ratio (Eppendorf BioPhotometer, Eppendorf Iberica, Madrid, Spain). cDNA synthesis and qPCR analysis were performed as described earlier (Maillo *et al.* 2016) in accordance with MIQE guidelines (Bustin *et al.* 2009). Briefly, RT reaction was carried out following the manufacturer's instructions (Epicentre Technologies Corp., Madison, USA) using poly (T) primers, random primers and MMLV high-performance reverse transcriptase enzyme in a total volume of 50 μL to prime the RT reaction and to produce cDNA. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 50 units of reverse transcriptase. Afterward, they were incubated at 25°C for 10 min to favor the annealing of random primers, followed by 37°C 60 min to allow the RT of RNA, and finally 85°C 5 min to denature the enzyme.

Primers (Table 1) were designed using Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primerblast/) to span exon–exon boundaries when possible. All qPCR reactions were carried out in duplicate on the Rotor Gene 6000 Real Time Cycler TM (Corbett Research, Sydney, Australia) by adding 2 μL aliquot of each sample to the PCR mix (GoTaq qPCR Master Mix, Promega) containing the specific primers selected to amplify the genes listed in Table 1. Cycling conditions were 94°C for 3 min followed by 35 cycles of 94°C for 15 s, 56°C for 30 s, 72°C for 10 and 10 s of fluorescence acquisition. Fold-changes in the relative gene expression of the target were determined using the equation $2^{-\Delta\Delta\text{CT}}$ (Livak & Schmittgen 2001) using *H2AZ*, *ACTB* and *GAPD* as endogenous controls.

Statistical analysis

Data are expressed as means \pm S.E.M. and were analyzed using IBM SPSS Statistics, version 23 for Windows. Gene expression data were analyzed using Sigma Stat (Jandel Scientific, San Rafael, CA, USA) software package. Mean differences in mRNA transcript abundance, spent medium carbohydrate metabolites, albumin flux data, TER data, FA transfer and FA transporter expression data among the experimental groups were compared with mixed-model ANOVA and post hoc Bonferroni tests including the fixed effect of treatment, the random effect of the repeat and their interaction (excluded when not significant). For normality and equality of variance reasons, pyruvate and lactate data were log transformed prior to statistical analyses. Differences with P values <0.05 were considered statistically significant.

Results

BOEC integrity and monolayer permeability

The TER measurements were expressed as 'relative TER increase' by comparing pre- and post-NEFA exposure measurements, as none of the treatments reduced TER to the extent that monolayer integrity was compromised (i.e. $<700\ \Omega\text{cm}^2$ (Chen *et al.* 2015)). Elevated NEFAs induced a significantly lower TER increase regardless of the exposure direction (Fig. 2).

The maximum FITC–albumin concentration in the non-supplemented compartment of unseeded wells was 4.3 μM . This flux was irrespective of assay direction and was correlated to the maximum FITC-albumin decrease in the opposite albumin-supplemented compartment. The maximum flux (i.e. concentration of FITC-albumin in the non-supplemented compartment) observed in the unseeded wells was therefore maximum 28.67% of the initial FITC-albumin concentration in the supplemented compartments at the beginning of the assay (i.e. 15 μM). Regardless of the treatment, when basal to apical flux was observed in seeded wells, the maximum FITC-albumin concentration in the non-supplemented compartment of the control wells was 0.51 μM (or 3.4%). When apical-to-basal flux was observed, the maximum flux was 1.8% of the initial FITC-albumin at the beginning of the assay

Table 1 List of primers used showing primer sequences, fragment sizes, and gene bank accession numbers. GAPDH, H2AFZ and ACTB were used as endogenous controls.

Gene	Gene name	Primer sequence (5'–3')	Fragment size (bp)	Gene bank accession no.
ACACA	Acetyl-CoA carboxylase alpha	AAGCAATGGATGAACCTTCTTC GATGCCCAAGTCAGAGAGC	196	FN185963.1
ACSL1	Acyl-CoA synthetase long-chain family member 1	TGACTGTTGCTGGAGACTGG TGTGCTTCTCTGTCGATG	250	NM_001076085.1
ACTB	Actin, beta	GAGAAGCTCTGCTACGTCG CCAGACAGCACCGTGTGG	264	AF191490.1
BAX	BCL2-associated X protein	CTGGAGCAGGTGCCTCAGGA ATCTCGAAGGAAGTCCAGCGTC	300	NM_001166486.1
BCL2	B-Cell CLL/lymphoma 2	GGAGCTGGTGGTTGACTTTC CTAGGTGGTCATTGAGGTAAG	517	BC147863.1
CAV1	Caveolin 1	TCAGCCGTGTCTATTCC ATTTCTTTCTGCGTGTTG	103	NM_174004.3
CD36	CD36 molecule, fatty acid translocase	GCTCCTTAAGCATTCTTGGAT CACCAGTGCAACGCATTT	151	NM_001278621.1
CPT1B	Carnitine palmitoyltransferase 1B	CTGCCCCGCTGGGAAATGCTGT CAGTCTCTCCTCCCCGGGCTGG	332	NM_001034349.2
ESR1	Estrogen receptor 1	CCCGCAAGGTTCTGAGAATCC CAAGGCGTGCCACGTAGAAGT	159	NM_001001443.1
FABP3	Fatty acid binding protein 3	TTGTGCGGGAGATGGTTGA TGCCGAGTCCAGGAGTAGCC	147	NM_174313.2
FOXJ1	Forkhead box J1	AGCAAGGCCACCAAGATCACC CCGAGGCACCTTGATGAAGCAC	145	NM_001192076.1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	ACCAGAAGACTGTGGATGG ATGCCTGCTTACCACCTTC	247	NM_001034034.2
GPX1	Glutathione peroxidase 1	GCAACAGTTTTGGGCATCA CTCGCACTTTTCGAAGAGCATA	116	NM_174076.3
G6PD	Glucose-6-phosphate dehydrogenase	CGCTGGGACGGGTGCCCTTCATC CGCCAGGCCTCCCGCAGTTCATCA	347	NM_001244135.1
H2AFZ	H2A histone family, member Z	AGGACGACTAGCCATGGACGTGTG CCACCACCAGCAATTGTAGCCTTG	209	NM_174809
LDHA	Lactate dehydrogenase A	TTCTTAAGGAAGAACATGTC TTCACGTTACGCTGGACCAA	310	NM_174099.2
LPL	Lipoprotein lipase	ATTGCTCAGCATGGCTCGGAAG TCCAGGGCCATACACTGACTG	309	NM_001075120.1
OVGP1	Oviductal glycoprotein 1	AAGAATGAGGCCAGCTCAC TGCCGAAGATTTGGGGTCTC	219	NM_001080216.1
SHC1	SHC (Src homology 2 domain containing) transforming protein 1	GTGAGGTCTGGGGAGAAGC GGTTCGGACAAAGGATCACC	334	NM_001075305
SCL2A1	Solute carrier family 2 (facilitated glucose transporter) member 1 (former GLUT1)	CTGATCCTGGTCCGCTTCAT ACGTACATGGGCACAAAACCA	68	NM_174602.2
SOD1	Superoxide dismutase 1, soluble	ATCATTGGCCGACGATGGTG CCACAGGCCAAACGACTTCCAG	107	NM_174615
TJP1	Tight junction protein 1	AATCATCCGACTCCTCGTCCG CCCAAACACAGCGCGTAAAA	255	XM_010817146.1
TP53	Tumor protein P53	CTCAGTCCTCTGCCATACTA GGATCCAGGATAAGGTGAGC	364	NM_174201.2

as the maximum FITC-albumin concentration in the non-supplemented compartment of the control wells was 0.27 μM . Only apical NEFA significantly increased the proportion of FITC transfer (3.8%) across the membrane ($P < 0.05$, Fig. 3) compared to that in controls, and only in basal-to-apical assay direction. Overall, albumin flux from the basal to the apical compartment was approximately two times higher than that seen in the opposite direction. When the FITC transfer direction was inverted (to 'apical to basal'), no treatment effects could be observed ($P > 0.05$).

BOEC NEFA transfer capacity

Total FA concentration

In basal NEFA, a 19% (or $122.5 \pm 4.3 \mu\text{M}$) reduction of total FA content in the supplemented compartment could be detected after 24-h exposure. In parallel, there was a 21% (or merely $12.7 \pm 1.4 \mu\text{M}$) rise in FA content in the apical chamber compared to that in the initial concentrations. By contrast, in apical NEFA, total FA content fell by 53.4% ($334.2 \pm 28.2 \mu\text{M}$) with no FA transfer detected in the basal chamber.

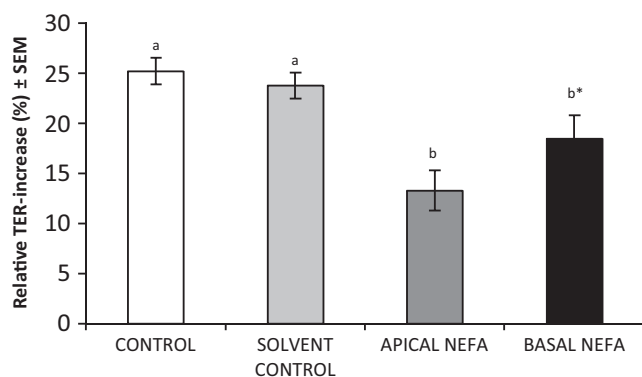


Figure 2 Relative TER increase was calculated through comparison of pre- and post-NEFA exposure TER measurements. ^{a,b}Different superscripts per bar indicate the statistical significant differences ($P < 0.05$); * $P = 0.05$.

FA profiling per FA fraction (free or esterified)

To specify the transfer, the total FA concentrations were separated in individual FAs and classified as free or bound, esterified FAs (triglycerides, cholesterol esters and phospholipids). For both apical and basal NEFA, significant differences in total FA could only be found in the free FA fraction. In basal NEFA, the significantly increased FAs in the non-supplemented, apical compartment were C16:0 ($56.0 \pm 20.0\%$, $P = 0.042$), C18:0 ($60.0 \pm 27.0\%$, $P = 0.098$) and C18:1 ($33.5 \pm 6.0\%$, $P = 0.082$) in the total FA-fraction, whereas in the free, unbound fraction, C14:0 ($58.0 \pm 27.8\%$, $P = 0.035$), C16:1-cis-9 ($81.1 \pm 19.3\%$, $P = 0.002$), C18:1-cis-9 ($72.2 \pm 3.9\%$, $P = 0.017$) and C18:1-cis-11 ($30.8 \pm 7.0\%$, $P = 0.004$) were found to be significantly increased.

In apical NEFA, no differences in FA increase could be detected in the non-supplemented compartment, as no FA transfer was observed ($P > 0.05$).

Intracellular lipid accumulation

Apical addition of NEFA caused an increased accumulation of neutral lipid droplets compared to other treatments (Fig. 4). Numerous lipid droplets were observed in the cytoplasm and distributed evenly across the BOEC monolayer. By contrast, when NEFA was added to the basal compartment, there was only limited lipid droplet accumulation in the BOECs. No lipid droplets were observed in the control groups.

BOEC fatty acid transporters

Fatty acid translocase/CD36 protein expression was upregulated in apical NEFA with 54.35 and 50.08% compared to that in basal NEFA and control conditions respectively ($P < 0.001$). Both APICAL and BASAL NEFA showed similar FABP3 expression and were upregulated compared to CONTROLS by an average of 58.15% ($P < 0.001$). CAV1 expression in apical NEFA

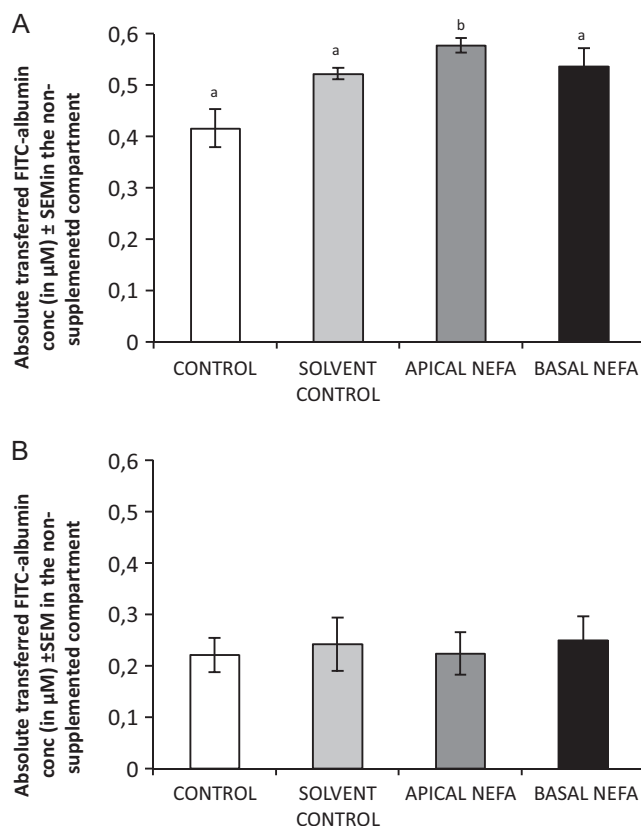


Figure 3 The permeability assay showed FITC-albumin flux measured in the non-supplemented compartment after a 3-h assay in which the FITC-albumin flux from the basal to the apical compartment (A) and the flux from the apical to the basal compartment (B) were observed. ^{a,b}Different superscripts per bar indicate statistically significant differences ($P < 0.05$).

was increased with 46.69% ($P < 0.001$) and 52.90% ($P < 0.001$) compared to that in basal NEFA and controls respectively (Fig. 5).

BOEC energy metabolism: glucose, lactate and pyruvate concentrations

Under untreated conditions, BOECs depleted 49.91 ± 3.61 nmol/well/h of glucose from the apical compartment and 55.54 ± 10.82 nmol/well/h from the basal compartment. In addition, 35.69 ± 5.04 nmol/well/h of pyruvate was depleted from the apical compartment and 38.87 ± 7.16 nmol/well/h from the basal compartment. BOECs released 141.21 ± 8.31 nmol/well/h of lactate into the apical chamber and 152.58 ± 5.33 nmol/well/h into the basal compartment.

Twenty four hours after the application of NEFA treatments, mean glucose release rose to 77.36 ± 3.54 nmol/well/h in the apical compartment and 139.26 ± 35.81 nmol/well/h in the basal chamber. Pyruvate depletion from the apical compartment was largely unchanged in response to NEFA addition (34.76 nmol/well/h), although depletion from the

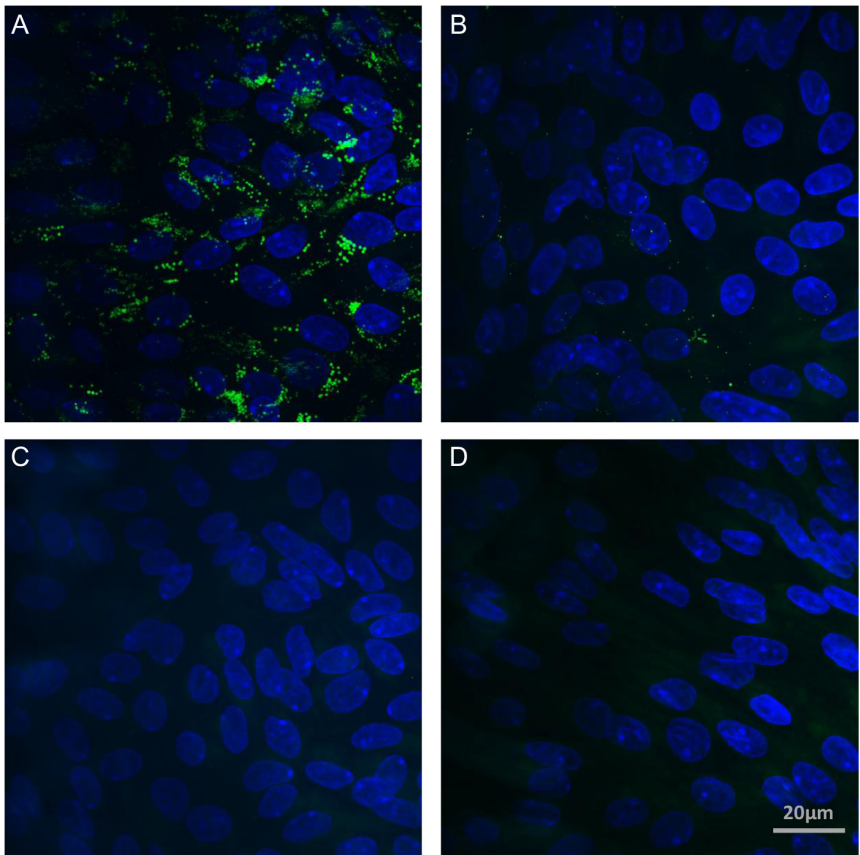


Figure 4 Lipid droplet analysis was performed using Bodipy 493/503 (green) to visualize intracytoplasmic droplets of neutral lipids and DAPI (blue) for staining nuclei. Monolayers from the control group (C) and solvent control (D) showed no lipid droplets, whereas BOEC monolayers from the apical NEFA group (A) clearly showed the accumulation of lipids in the cells, and BASAL NEFA (B) displayed little-to-no lipid droplets. Images were made at 60× magnification using confocal microscopy.

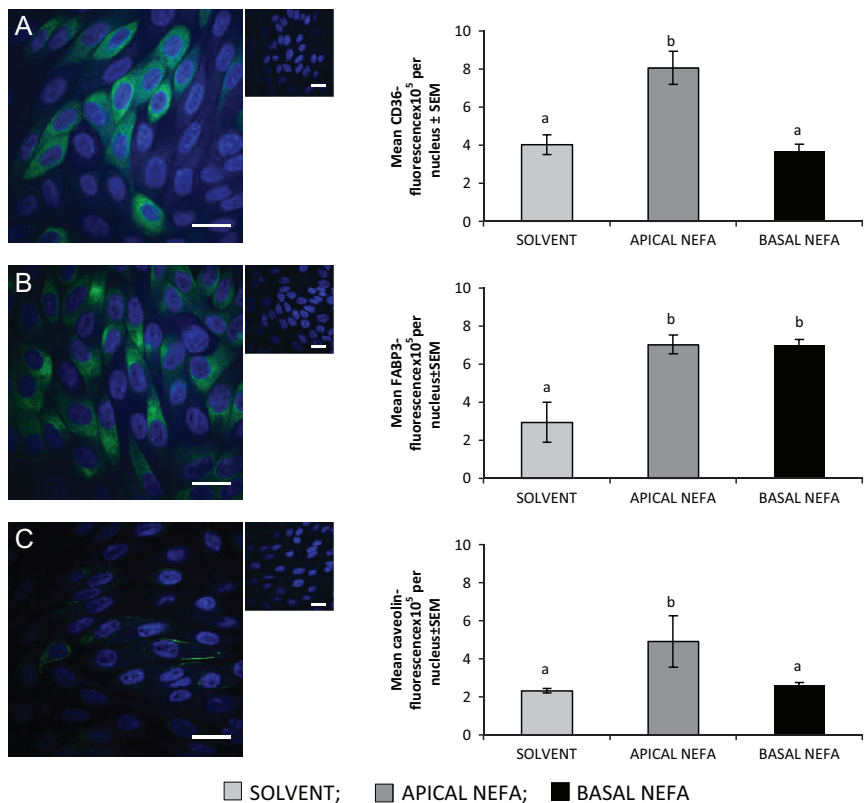


Figure 5 Immunolabelling of specific fatty acid transporters was performed to visualize and quantify specific FA transporter expression (green) per nucleus (blue). Fatty acid translocase CD36 (A), fatty acid-binding protein 3 (FABP3) (B) and caveolin 1 (CAV1) (C) are presented with their respective negative controls and mean transporter fluorescence per nucleus in different treatments. Scale bars indicate 20 µm. ^{a,b}Different superscripts per bar indicate statistically significant differences ($P < 0.05$).

basal compartment rose to 51.36 ± 8.34 nmol/well/h. Lactate appearance in the apical compartment was 154.92 ± 14.42 nmol/well/h and in the basal compartment was 190.32 ± 11.99 nmol/well/h (Fig. 6).

No differences among the treatments could be detected.

BOEC gene expression analysis

The effects of BOEC NEFA exposure on the expression profile of genes involved in apoptosis (Fig. 7A), oxidative stress and specific BOEC function (Fig. 7B)-related genes was subsequently investigated. Addition of NEFA to the apical compartment led to increased expression of *BCL2*, compared to basal addition and control groups ($P < 0.01$), the consequence of which

was to reduce the ratio of *BAX/BCL2* ($P < 0.01$). Stress adaptor protein, *SHC1*, was upregulated in response to apically administered NEFA ($P < 0.05$), although this was also apparent in the solvent control ($P < 0.01$). In addition, expression of *SOD1* was upregulated in response to apical administration of NEFA ($P < 0.05$). Expression of *OVGP1* (oviduct-specific glycoprotein expression), *ESR1* (estrogen receptor expression) and *FOXJ1* (ciliogenesis) were all unchanged in response to NEFA and regardless of the exposure direction. Next, the impact of NEFA exposure on genes related to energy metabolism (Fig. 7C and D) was examined. mRNA expression of *G6PD* was downregulated after addition of NEFA to the apical chamber ($P < 0.05$) but upregulated when NEFA was added to the basal compartment ($P < 0.05$). The expression of *CPT1B* ($P < 0.05$) and *ACSL1* ($P < 0.05$) transcripts were upregulated, whereas *ACACA* expression ($P < 0.05$) was decreased in response to apical addition of NEFA. Expression of BOEC FA transporters resulted in upregulated *CAV1* ($P < 0.001$) expression in APICAL NEFA compared to those in other treatments (Fig. 7E). Overall, fold-changes were low except for *BCL2* and *CAV1*.

Discussion

In this study, we hypothesized that elevated serum NEFA concentrations alter BOEC physiology, and more specifically, BOEC metabolism and barrier function, potentially affecting the zygote's micro-environment. Hereto, PCC system with hanging inserts was used to approach the BOECs in a most physiologically relevant manner (Fotheringham *et al.* 2011).

Overall, data indicate that APICAL NEFA resulted in an increased FITC-albumin flux from the basal to the apical compartment. This increased monolayer permeability was also associated with reduced monolayer growth as suggested by slower increasing TER values from Days 9 to 10. In basal NEFA, NEFA concentrations decreased in the basal compartment with a concomitant increase in the apical chamber indicating limited FA transfer. While apical FA administration resulted in the increased lipid droplet formation and no transfer to the basal compartment. Depletion of carbohydrate metabolites seemed mostly active in the basal compartment, regardless of the treatments, substantiating the distinct effects of cellular polarity on the use of energy substrates in the culture system. Furthermore, apical NEFA induced anti-apoptotic and anti-oxidative pathways as suggested by increased expression of *BCL2* and *SOD1* and may stimulate BOEC lipid metabolism through increased intracellular FA uptake (\uparrow *CAV1* and \uparrow FA-transporter protein expression *CD36*, *FABP3* and *CAV1*), and upregulation of *CPT1B* and *ACSL1*. To our knowledge, the present study is the first to attempt a deeper understanding in the characteristics of BOECs under the influence of elevated NEFAs, hereby confirming

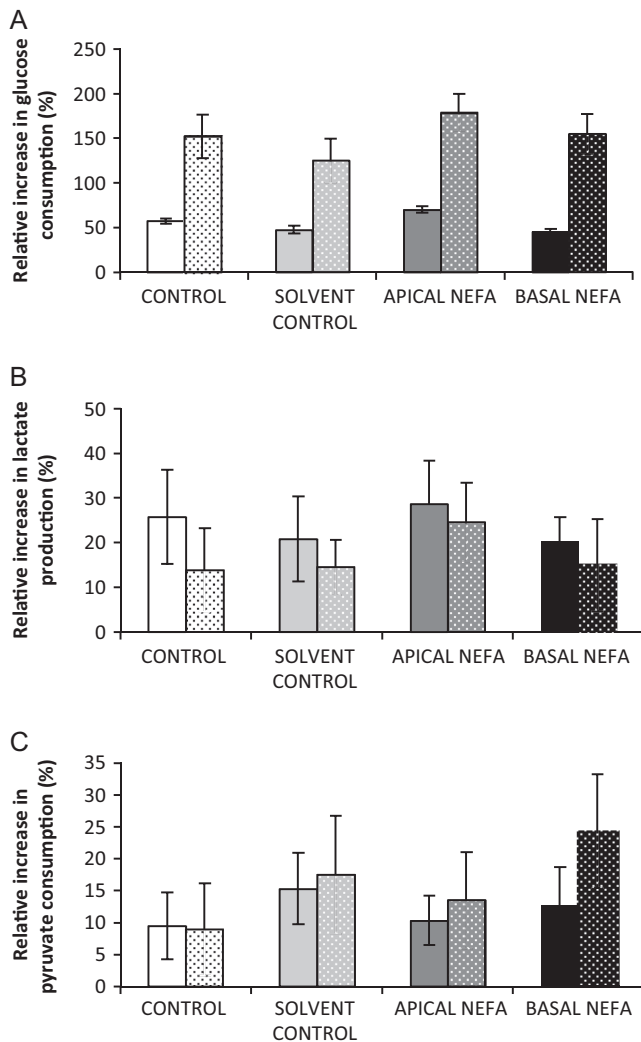


Figure 6 Glucose (A) and pyruvate (C) consumption and lactate (B) production (%) in spent medium were expressed as relative values of pre- and post-NEFA exposure samples taken with a 24-h interval and in different treatment groups. Full bars represent the apical compartment, and dotted bars represent the basal compartment.

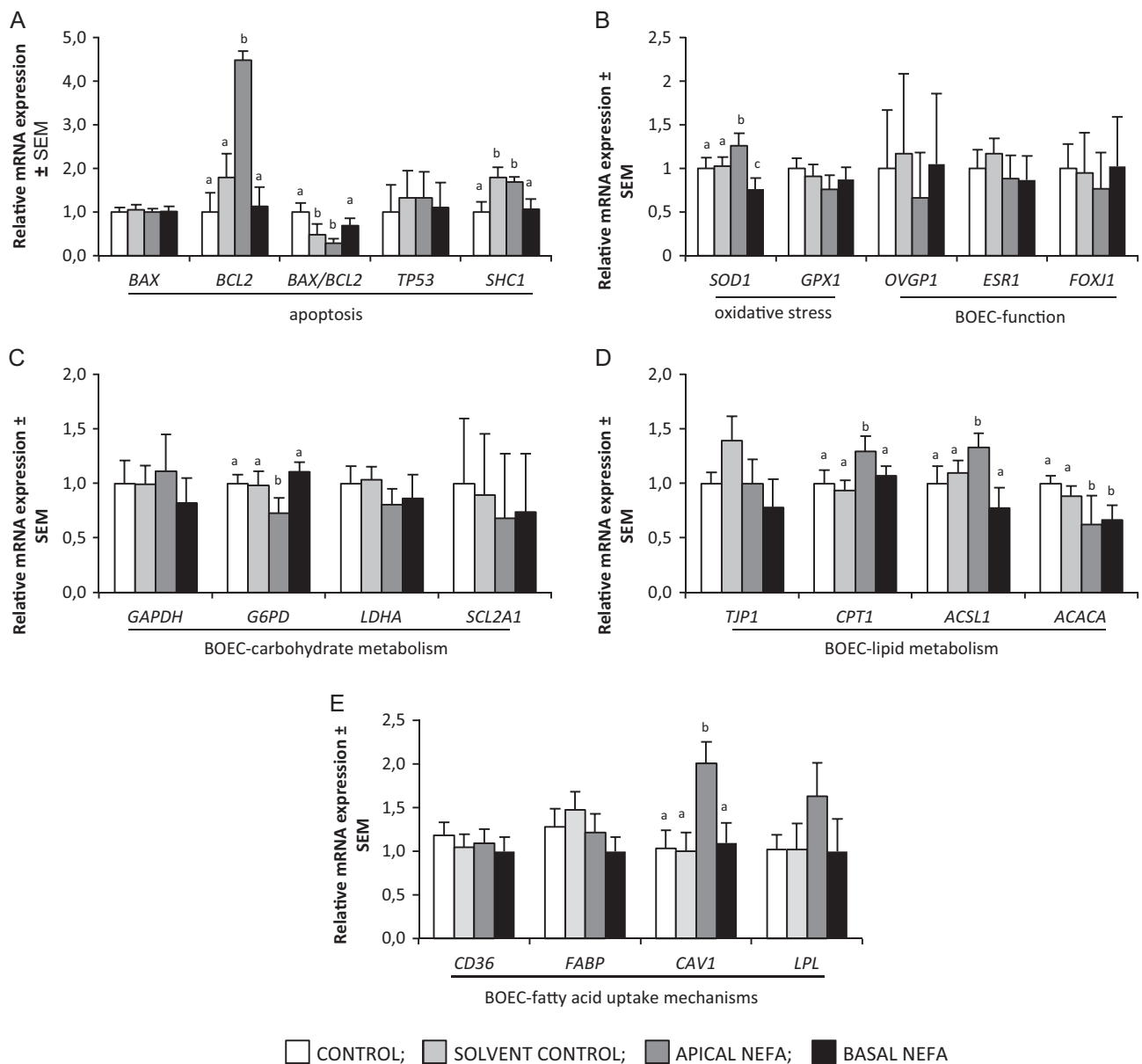


Figure 7 mRNA transcript abundance (\pm S.E.M.) after qRT-PCR gene expression analyses. Genes are sorted based on function: (A) apoptosis, (B) oxidative stress and BOEC function, (C) BOEC carbohydrate metabolism, (D) BOEC lipid metabolism and (E) FA uptake. ^{a,b,c}Different superscripts per bar indicate statistically significant differences ($P < 0.05$).

cell polarity within the culture system and localizing different FA transporters.

Characterization of monolayer integrity by means of TER measurements resulted in ongoing increase in TER values during the NEFA exposure period due to continuous cell growth (Jordaens *et al.* 2015). In rat mammary epithelium, similar effects were observed and were considered to result from palmitic and stearic acid exposure (Wicha *et al.* 1979). Data furthermore indicated that apical NEFA resulted in reduced TER increase during the 24-h NEFA exposure period and therefore decreased the tightness of intercellular cell contact (Chen *et al.* 2015). These data were supported

by the monolayer permeability assessment using FITC-labeled albumin. Here, only apical NEFA resulted in an increased FITC-albumin flux suggesting increased monolayer permeability and reduced monolayer tight junction quality (Anderson & Van Itallie 2009) in this treatment. Earlier, Roche and coworkers (Roche *et al.* 2001) made similar observations in Caco2-cells and reported that both TER permeability and expression of tight junctions decreased due to elevated FAs and the tight junction modulating capacity of NEFAs. Considering the apical positioning of tight junctions between adjacent cells, the increased monolayer permeability in apical NEFA of our study, may result from a more intense

NEFA/tight junction contact in this treatment. The effects on permeability here observed, however, were limited specifically to the basal-to-apical albumin flux only. When the assay direction was inverted, total flux did not show any treatment effects. Apical-to-basal albumin flux was, however, lower compared to basal-to-apical flux, suggesting the oviductal lining to be still intact. It may also suggest intracellular uptake of the albumin in the apical-to-basal assay direction considering the equal amounts of albumin decrease in the supplemented compartments (Fig. 8) in both assay directions, which can be explained by the expression of albumin-binding cell surface receptors on the apical cell side of the oviduct only (Argaves & Morales 2004).

These findings and unpublished fluorescence microscopic imaging may therefore question the accuracy of the apical-to-basal assay direction as permeability parameter, but do provide us with interesting considerations when assessing the FA transfer data.

FA transfer across the BOEC monolayers in basal NEFA showed a 19.5% FA decrease in the supplemented, basal compartment and a 21.1% increase in the opposite,

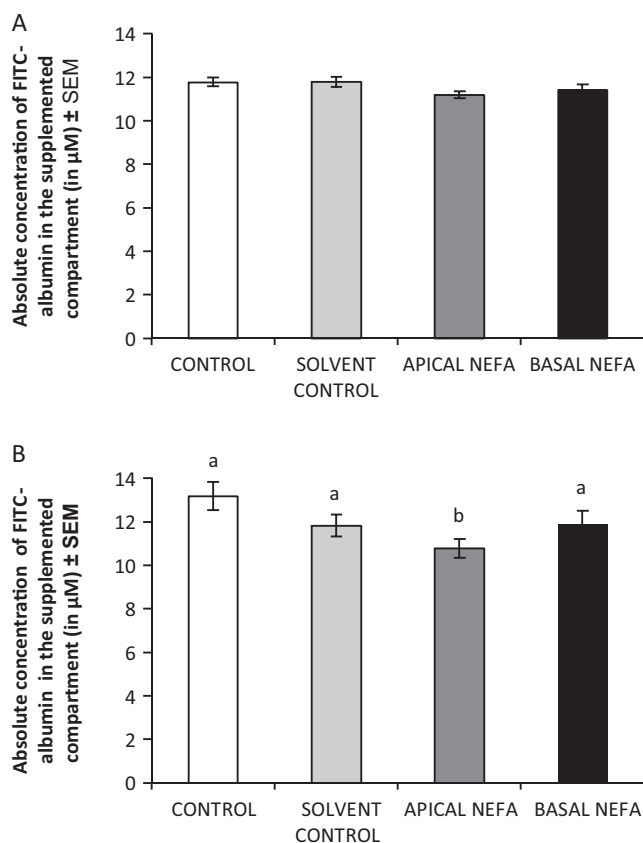


Figure 8 The permeability assay showed a decrease in FITC-albumin concentrations measured in the albumin-supplemented compartment after a 3-h assay in both the basal-to-apical (A) and apical-to-basal (B) assay direction. ^{a,b}Different superscripts per bar indicate statistically significant differences ($P < 0.05$).

apical compartment. The absolute values of the transfer, however, suggest that only a minor proportion of the FAs are transferred from the basal-to-the apical compartment and substantiate the concept of a potential gatekeeper function of the oviduct. Intervening with transfer of detrimental metabolites to the oviductal lumen may thus be considered as an embryoprotective mechanism. When observing the transferred FAs in closer detail, we determined that all transferred FAs were unbound FAs and that it was mostly oleic and palmitic acid that could be transferred to the apical compartment. These data indicate that FA transfer might be a selective process with a distinct active component for FA uptake (Glatz *et al.* 2010). Gas chromatographic analysis also revealed non-supplemented FAs to be present in the luminal chamber, suggesting some degree of metabolic modification. For example, the presence of C14:0, which was not added basally, may be indicative for *de novo* synthesis or conversion of C16:0 and C18:0 through partial oxidation (Lopaschuk *et al.* 2010). The presence C16:1-cis-9 may be a consequence of desaturation of PA and C18:1-cis-11 from elongation (Jakobsson *et al.* 2006). Therefore, not only transfer but also FA metabolism could be detected in this treatment (Fig. 9 for conceptualization). In contrast to this, when NEFA was added to the apical chamber, there was no subsequent appearance in the basal compartment. The FA concentration in the apical supplemented compartment did, however, decrease by more than 50% over a 24-h timespan. The reduced apical FA concentration, without signs of FA transfer, suggests intracellular FA uptake for storage in lipid droplets. Indeed, an increased accumulation of cytoplasmic lipid droplets in BOECs was observed in this treatment using Bodipy staining. The differences in lipid accumulation between the treatments were so apparent that no further quantification steps were undertaken. Cnop and coworkers (Cnop *et al.* 2001) observed lipid droplet formation in rat pancreatic cells and suggested cellular triglyceride accumulation as a cytoprotective mechanism against FA-induced lipotoxicity. In our current data, FA deposition in neutral lipid droplets was most abundantly observed in apical NEFA. Basal NEFA showed lipid droplets to a limited extent, whereas lipid accumulation was completely absent in the control and solvent control. On the basal cell side, FABPs require non-albumin-bound FAs for intracellular FA uptake, which requires lipoprotein lipases that are typically expressed by endothelium (Glatz *et al.* 2010). These lipases are not present in our experimental design and may elucidate the lack of lipid accumulation in BASAL NEFA as most supplemented NEFAs in our experiments are albumin bound. The apical cell side, on the other hand, and as mentioned previously, typically expresses caveolins, megalins, cubilins and lipoproteins allowing albumin-bound FA endocytosis (Moestrup & Verroust 2001, Argaves & Morales 2004), facilitating cellular uptake of NEFA/albumin complexes from the luminal

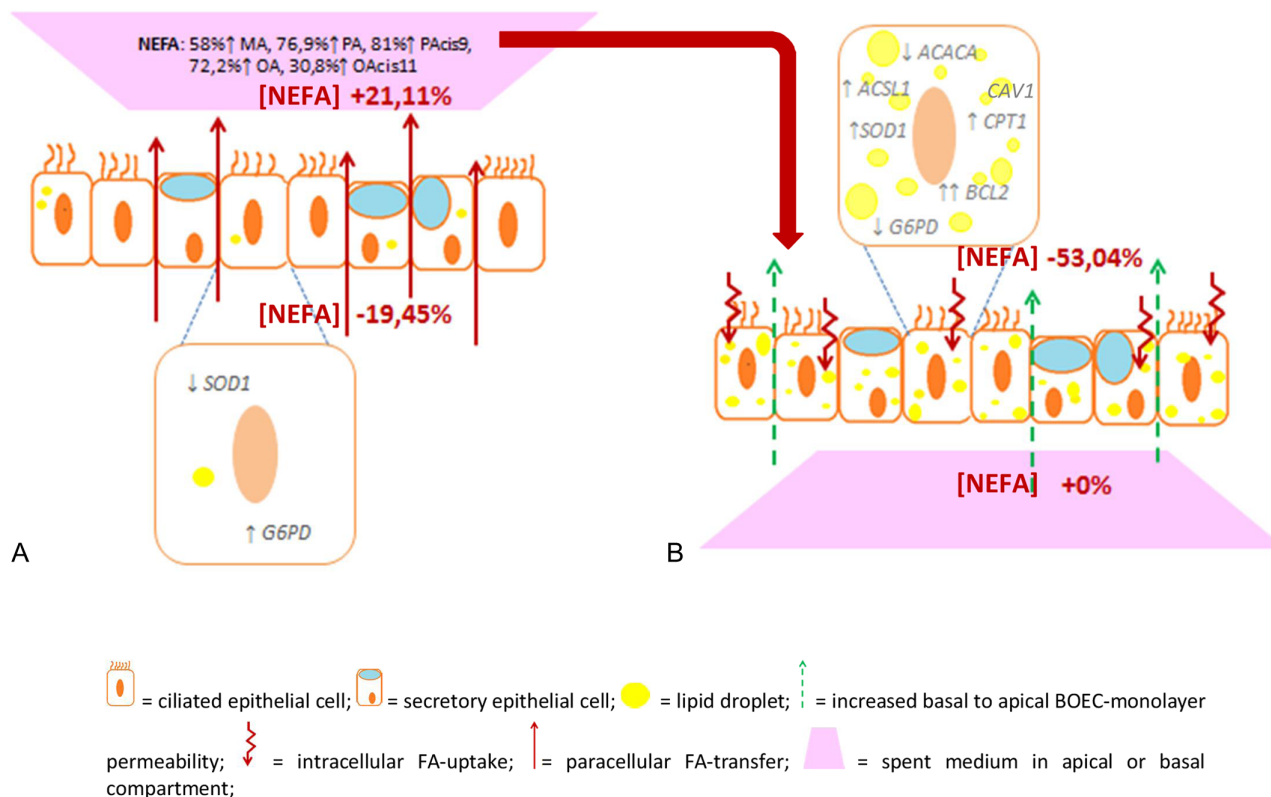


Figure 9 Graphic summary of the obtained results and conceptualization of fatty acid transfer across BOEC monolayers as suggested in the experiments previously. Red arrows indicate the FA transfer: via paracellular transport the basally supplemented FAs (A) are directed to the apical oviductal lumen compartment as non-esterified FAs, where they can be internalized by the cells and directly used as an energy substrate or in case of abundance, stored in lipid droplets (B). Gene expression is altered to allow the cells metabolic adaptation and upregulated anti-oxidative and anti-apoptotic pathways after exposure to elevated NEFAs. Green arrows suggest the increased permeability from the basal to the apical compartment in apical NEFA, proposing the possibility that the oviductal micro-environment may be subjected to all kinds of metabolic changes. Nuclei are orange, secretory granules are blue and lipid droplets are depicted in yellow. These data suggest cellular adaptation to changing environmental conditions.

chamber in our experimental setting. The presence (and abundance) of these transporters was confirmed in the current *in vitro* model through immunolabelling of BOEC FA transporters and may elucidate the quantitative difference in lipid droplets observed between treatments. In this respect, *CAV1* mRNA transcript abundance was upregulated in apical NEFA compared to other treatments, which resulted in increased translation of *CAV1*. *FAT/CD36* and *FABP3* showed similar FA transporter expression in APICAL NEFA; however, no differences in mRNA transcripts could be detected when comparing different treatments. The latter might be explained by the increased use of the transcripts for translation with limited *de novo* transcriptional activity during the period investigated (24h) as seen in early embryos (Robert 2010). Interestingly, *CD36* transporters are typically expressed in tissues that favor high FA metabolism, as seen in mammary glands (Spitsberg *et al.* 1995), and also, metabolic conditions have shown to alter FA utilization and FA transporter expression as observed in adipocytes of diabetic rats (Berk *et al.* 1997) and as simulated in the current experimental setting.

The fact that yet a few lipid droplets could be detected in basal NEFA can be accounted to the limited FA transfer to the apical compartment in this treatment. Furthermore, these observations suit our findings made in the permeability assay where intracellular albumin uptake could only be observed when the fluorescent albumin was supplemented in the apical compartment. Complementary to these findings, basal NEFA treatment resulted in little-to-no differences in mRNA transcript abundance in the selected genes regarding lipid metabolism, possibly since the administered FAs are not taken up or partly redirected to the apical compartment. Gene expression analysis in apical NEFA, otherwise, resulted in increased lipid oxidation and reduced lipid synthesis. These data are suggestive for increased lipid metabolism of BOECs. However, and due to the abundance of the supplemented FAs, the supply may surpass the FA metabolism rate. Hereto, lipid storage may be employed as an adaptive tool to fulfill mitochondrial energy supply without hindering the redox status and by reducing the amount of lipotoxic intermediates (Aon *et al.* 2014). This mechanism not only

protects the cells from NEFAs detrimental effects but also may 'purify' the oviductal microenvironment. The environmental conditions for optimal embryo growth can thus be significantly improved, which is crucial considering the critical changes the embryo undergoes during its stay in the oviduct (Latham & Schultz 2001, Inbar-Feigenberg *et al.* 2013).

Analysis of spent medium for BOEC-carbohydrate metabolites did not reveal any significant differences in the consumption of glucose or pyruvate or production of lactate. In this respect, data are consistent with BOEC transcriptome data. Regarding the genes selected for the assessment of BOEC energy metabolism, only *G6PD* transcript abundance showed significant differences: *G6PD* was downregulated in apical NEFA and upregulated in basal NEFA; however, none of the other glucose metabolism-related genes were affected. This suggests that glucose may be increasingly directed toward the pentose phosphate pathway in basal NEFA, but the overall consumption was not affected. Regardless of the treatment, glucose uptake was most apparent in the basal, serum compartment, shifting glucose metabolites such as lactate in the apical compartment. *In vivo* BOECs are also provided with glucose via the serum (Leese 1988): our findings therefore support the natural conditions. Earlier experiments (Jordaens *et al.* 2015), however, indicated that during the 24-h exposure window, BOEC monolayers showed continuous growth. In the current study, similar effects have been observed in increasing TER values and elevated post-exposure glucose consumption in control groups. The latter was therefore normalized using pre-exposure data from the controls to minimize false interpretation. BOEC monolayers also showed an altered mitotic capacity, altered migration capacity and modified functionality due to elevated NEFAs (Jordaens *et al.* 2015), which may easily mask the turnover differences. In other cell types, similar NEFA effects have been observed. Rat hepatocytes showed increased apoptosis due to steatosis after OA and PA exposure (Ricchi *et al.* 2009), pancreatic B-cells in rats were hyperplastic with morphological abnormalities under the influence of FAs (Milburn *et al.* 1995) and mouse embryos lacked cell proliferation capacity and showed a reduced developmental competence (Nonogaki *et al.* 1994). Interpretation of the current glucose, pyruvate and lactate turnover data should therefore be done with caution as the NEFA conditions are known to affect monolayer characteristics. This is further supported by the data on *gene expression* as we observed increased expression of genes related to FA uptake, *CAV1* in apical NEFA. Caveolins are membrane proteins, typically expressed at the apical cell side, involved in clathrin-independent endocytosis of proteins and lipids (Nabi & Le 2003). Upregulation of these proteins in the presence of FA abundance may elucidate the increased intracellular lipid uptake. Furthermore, upregulation

of lipid metabolism β -oxidation (\uparrow *CPT1* and *ACSL1*) in this treatment, with the downregulation of FA synthesis-related genes (\downarrow *ACACA*) appears to confirm our theory on embryoprotective 'purification' of the oviduct microenvironment. The excess of FAs presented to the cells apically may therefore be consumed as a metabolic fuel, whereas *de novo* FA synthesis may be limited (Aon *et al.* 2014). In most tissues, *de novo* FA synthesis is of minor importance as the cellular requirements are predominantly met through FA supply via the blood. Increased levels of circulating FAs inhibit FA synthesis (Weis *et al.* 1986) and may result in decreased transcriptional activity of *ACACA*, as seen in the current data. The excessive FA oxidation may also prompt to increase oxidative stress (Aon *et al.* 2014) in BOECs. The upregulation of *BCL2* and *SOD1* in apical NEFA may therefore be a direct reaction of BOECs to NEFA exposure, increasing the cells' anti-oxidative and anti-apoptotic capacity. Harvey and coworkers (Harvey *et al.* 1995) and Tse and coworkers (Tse *et al.* 2008) made similar observations in embryo/BOEC co-cultures and may further explain BOEC's embryoprotective capacity.

Ultimately, our findings may contribute to the concept that elevated NEFAs may modify the composition of the oviduct luminal fluid and alter the pre-implantation embryo's microenvironment. However, specific modifications were made to the experimental design. In this respect, NEFA exposure was limited to 24h, whereas *in vivo* NEFA concentrations are elevated over longer periods (Butler *et al.* 2003). Prolonged *in vitro* FA incubation was, however, in other cell types, associated with a significant decrease in cell viability (Ricchi *et al.* 2009). Furthermore, even acute NEFA exposure induced negative effects on BOECs and remains the only option to solely observe the direct cellular effects of NEFAs. In addition, the use of serum for optimal cell attachment compromised the definition of the culture conditions. To minimize the serum effects, concentrations were contained to 5%, and the serum was analyzed for NEFA content prior to use. Further *in vivo* studies are required to investigate the changes in the oviduct luminal fluid associated with maternal metabolic disorders and how they may affect the micro-environment of the pre-implantation embryo. Nonetheless, our *in vitro* findings provide novel insights into the understanding of oviduct interactions with FAs.

In conclusion, elevated NEFAs affect BOEC metabolism and barrier function in a polarity-dependent manner. In this respect, BOECs in basal NEFA potentially shield the luminal environment from elevated NEFAs by allowing only a limited amount of FAs to be transferred from the basal to the apical compartment. BOECs in APICAL NEFA may clear the micro-environment of the pre-implantation embryo from NEFAs through increased monolayer permeability, intracellular lipid accumulation and FA metabolism. Overall, the oviduct may modulate

its micro-environment in favor of the early embryo by alleviating the potential lipotoxic effects.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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