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Metabolic-hypoxic modulation of cytokine induction of intestinal endothelial adhesion molecules: Relevance to ischemic injury mediated necrotizing enterocolitis?

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ABSTRACT

Background: Necrotizing enterocolitis (NEC) triggers an intense inflammatory response in the neonatal gut associated with cytokine activation, altered nutrient status and intracellular O_2-deprivation. Endothelial cell adhesion molecules (ECAMs) play critical roles in driving immune cell infiltration into inflamed gut. Currently, relationships between inflammation, metabolism and ECAM expression remain poorly understood in NEC. We studied the effects of metabolic depletion (aglycemia/ hypoxia) on TNF-α mediated ECAM expression including ICAM-1, MAdCAM-1, VCAM-1 and E-selectin, in vitro in intestinal microvascular endothelial cells (IMEC).
Methods: To study the effects of TNF-α, aglycemia and hypoxia (alone or in combination) IMECs expression of adhesion molecules was studied using cell surface ELISA and immunoblotting.
Results: Total VCAM-1 expression was induced TNF-α and by hypoxia + TNF-α, cell surface expression was induced by hypoxia, TNF-α, TNF- α+hypoxia, and TNF- α+hypoxia and aglycemia. Total ICAM-1 increased following TNF- α, TNF- α+hypoxia, hypoxia + aglycemia, and TNF- α+hypoxia + aglycemia. Total MAdCAM-1 protein expression was significantly induced by a combination of TNF-α+hypoxia + aglycemia and cell surface expression induced by TNF- α+hypoxia. Surface expression of E-selectin was induced by TNF- α+aglycemia and TNF- α+hypoxia + aglycemia.
Conclusion: Energy metabolism influences inflammation induced injury through mobilization of intestinal ECAMs, and may represent an important mechanism in NEC pathology.

1. Background

Necrotizing enterocolitis (NEC) is a common disease in neonates, especially in infants with birth weight <1500 g. A study by the NICHD (National Institute of Child health and Human Development) Neonatal Research Network, reported NEC in 4–11.5% of all premature births, with the highest incidence in infants weighing <750 g [1]. We recently described the pathophysiology of NEC with its manifold clinical challenges, epidemiology, and management strategies [2]. The clinical and histopathological features of NEC are provoked by tissue ischemia, abnormal proliferation of gut flora, intensified inflammatory responses, and possibly aggressive enteral feeding all of which contribute to its pathogenesis [2,3].

Although significant advances have been made in the survival and outcomes of premature infants, NEC remains a challenging cause of morbidity and mortality and its etiology enigmatic. Clinical and animal studies suggest that mucosal injury triggers a cascade of events which contribute to the pathogenesis of NEC. Several reports have attributed NEC to an intestinal ischemic-reperfusion injury in the postnatal period [2,3]. More than 90% cases of NEC are observed after enteral feeding [4], suggesting that feeding drives proliferation of the intestinal bacteria, which invade the mucosa through damage to the mucosal epithelial barrier3]. Invading bacteria induce inflammation by releasing chemoattractant formyl peptides which engage formyl peptide receptors and toll-like receptors (TLRs) [5–7], leading to leukocyte infiltration. The release of cytokines like IL-1β and TNF-α by resident and tissue infiltrating macrophages, mast cells and lymphocytes

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activates expression of endothelial adhesion molecules such as ICAM-1, VCAM-1, MAdCAM-1 and E- and P -selectins. TNF-α also disturbs endothelial and epithelial barriers by disrupting tight junctions, further driving the infiltration and recruitment of inflammatory cells, altering epidermal growth factor signaling and cell death all of which create the gut pathology seen in NEC [8–15].

In this setting, endothelial cells may act as a "gate keeper" against inflammatory cells penetration, controlling leukocyte binding and extravasation under normal and pathological states. Microvascular inflammatory responses involve sequential leukocyte-endothelial cell interactions including rolling, adhesion, activation and leukocyte emigration mediated by selectin and immunoglobulin family members on endothelium and leukocytes [16]. Investigators have studied the roles of ECAMs using blocking monoclonal antibodies or genetically engineered ECAM-deficient mice to validate specific ECAMs in features of the pathogenesis of several acute and chronic inflammatory disorders [17,18]. So far, no studies have examined how metabolic stress, which often exists in NEC, modulates cytokine-mediated expression of intestinal ECAMs. Here we examined how metabolic stresses might alter cytokine induced intestinal inflammation often seen in NEC.

1.1. Objectives

We studied how expression of Intercellular Adhesion Molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule -1 (VCAM-1), Mucosal Vascular Addressin Cell Adhesion Molecule (MAdCAM-1) and E-selectin were altered by TNF-α, under conditions of aglycemia and hypoxia using cultured murine intestinal microvascular endothelial cells (IMECs).

2. Materials and methods

2.1. Cell culture

IMECs were cultured from 'Immortomouse', H-2 K(b)tsA58 in DMEM with 10% fetal calf serum (FCS) and 1% Penicillin/Streptomycin/Amphotericin as previously described [19].

2.2. Antibodies and dilutions

ICAM-1 (Cat. No. 09351D) was purchased from Pharmingen, San Diego, CA; VCAM-1 (Cat. No. 553329), MAdCAM-1 (MECA-367, Cat. No. 09721D) E-selectin (Cat. No. 553748) were purchased from BD Biosciences, San Diego, CA. Antibodies were used at the following dilutions: VCAM-1 1:1000- ELISA, 1:500- immunoblot, ICAM-1 1:1000- ELISA and immunoblot, MAdCAM-1 1:1000- ELISA and immunoblot and E-selectin 1:1000-ELISA and immunoblot.

3. Treatment protocols

IMEC were cultured in the central 24 wells of 48 wells plates for cell ELISA and in 6 wells plates for immunoblotting studies. All cells were cultured in 10% DMEM at 33 °C. At confluency, cells were switched for at least 6 h to media consisting of 10% DMEM for 18 h at 37 °C. After 24 h at 37 °C cell surface ELISA and immunoblotting studies were performed as described below.

3.1. Aglycemia

At confluency, cells were subjected to aglycemia using glucose free DMEM, supplemented with 1% HEPES, sodium bicarbonate (3.7gms/ml, pH 7.35) and 1% FCS. Cells were either incubated in 10% DMEM or aglycemic buffer, with and without TNF-α.

3.2. Hypoxia

Cells were subjected to hypoxia conditions by placing them in 10% DMEM and incubating in hypoxic (1% O2, 5% CO2 and 94% N) conditions for 6 h. Cells were maintained hypoxic or normoxic in medium consisting of 10% DMEM with or without TNF-α.

3.3. Hypoxia + aglycemia

Cells were subjected to combined hypoxia + aglycemia by placing cells in glucose free buffer±TNF-α and incubating them in a hypoxic atmosphere for 6 h.

3.4. Cell surface ELISA

Following treatments, cells were fixed in 1% buffered formalin (PFA) for 5 min followed by 3 washes in DPBS/HBSS/1%FCS. Cells were incubated in the primary antibody (prepared in DPBS/HBSS/5%FCS) for 30 min. at 37 °C, washed and incubated in peroxidase conjugated secondary antibody (Sigma, 1:1000) for 30 min. at 37 °C. Cells were washed and then incubated with tetra methyl benzidine. Chromogen reaction was stopped with 75ul of 8 N H_2SO_4 and read on multiscan plate reader (MTX Lab systems, Inc., Vienna, Virginia) at 450 nm.

3.5. Immunoblotting

Protein was extracted using non-reducing buffer and a protease inhibitor cocktail tablet (Roche), sonicated and denatured at 100 °C for 3 min., resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membrane. Membranes were then immunoblotted ICAM-1, VCAM-1, MAdCAM-1, E-selectin and P-selectin antibodies and developed using chemiluminiscence as previously described. Immunoblot band integrated densities were measured via "NIH-Image J" (version 1.46 r).

3.6. (3,4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

IMECs were grown in 96 well plates and treated as described above. After incubation, culture medium was removed and cells washed with HBSS and incubated with phenol-red and serum free RPMI containing (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Plates were incubated for 1 h at 37 °C and reduced MTT extracted by addition of acidified isopropanol. The absorbance was read at 570 nm (subtracting background at 650 nm).

3.7. Crystal violet staining

To quantify cell adhesion, crystal violet staining was used. IMECs were grown in 96 well plates and treated as described. Culture medium was then aspirated, cells fixed in PFA and stained with 50 μl of 1% crystal violet for 30 min. and washed with PBS. Absorbance at 540 nm was recorded.

3.8. Statistical analysis

One-way ANOVA with Dunnett's post-hoc testing was performed using Instat3 software (Graphpad instat3, La Jolla, CA) to check significance. All values are expressed in ± SEM. Values of $^{*}p < 0.05$ were considered significant.

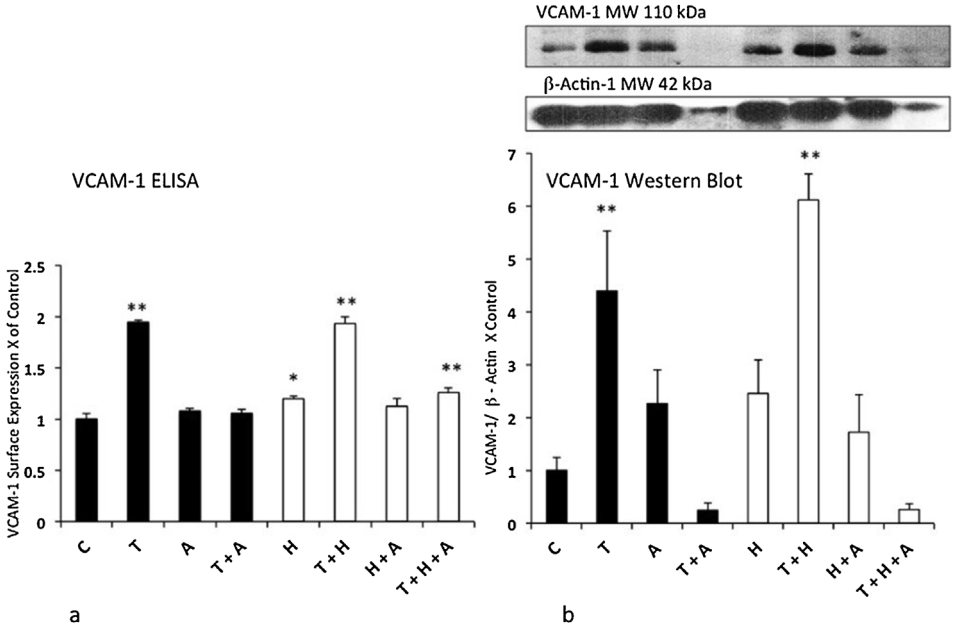

Fig. 1. a) Surface expression of VCAM-1 molecule under 8 different conditions including C = Control, T = TNF-α, A = Aglycemia, T + A= TNF-α and Aglycemia, H= Hypoxia, T+H = TNF-α and Hypoxia, H + A = hypoxia + aglycemia, T+ H + A = TNF-α and hypoxia + aglycemia. *n = 8, *p < 0.05, ** p < 0.01, *** p < 0.001*. b) Immunoblotting of VCAM-1 normalized with β -actin with 8 different treatment conditions including C = Control, T = TNF-α, A = Aglycemia, A + T = TNF-α and Aglycemia, H= Hypoxia, H + T = TNF-α and Hypoxia, H + A = hypoxia + aglycemia, T+ H + A = TNF-α and hypoxia + aglycemia. *n = 3, *p < 0.05, ** p < 0.01, *** p < 0.001.*

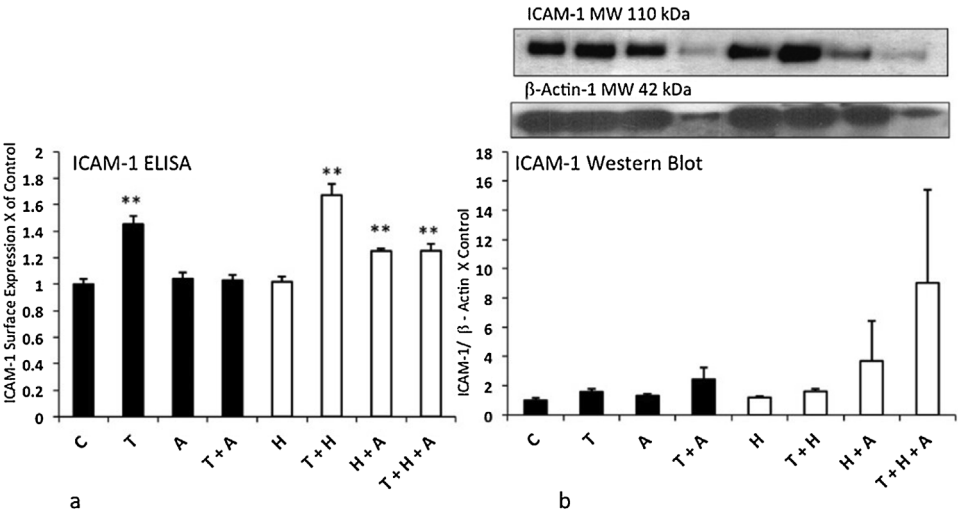

Fig. 2. a) Surface expression of ICAM-1 molecule under 8 different conditions including C = Control, T = TNF-α, A = Aglycemia, A + T = TNF-α and Aglycemia, H= Hypoxia, H + T = TNF-α and Hypoxia, H + A = hypoxia + aglycemia, T+ H + A = TNF-α and hypoxia + aglycemia. *n = 8, *p < 0.05, ** p < 0.01, *** p < 0.001*. b) Immunoblotting of ICAM-1 normalized with β -actin with 8 different treatment conditions including C = Control, T = TNF-α, A = Aglycemia, A + T = TNF-α and Aglycemia, H= Hypoxia, H + T = TNF-α and Hypoxia, H + A = hypoxia + aglycemia, T+ H + A = TNF-α and hypoxia + aglycemia. *n = 3, *p < 0.05, ** p < 0.01, *** p < 0.001.*

4. Results

4.1. VCAM-1 ELISA

TNF- α and combined hypoxia + TNF- α induced a near 2X increase in VCAM-1 compared to control ($p < 0.01$); Significant expression of VCAM-1 was observed with hypoxia alone ($p < 0.05$). With hypoxia + aglycemia + TNF- α also showed significant exacerbation of VCAM-1 ($p < 0.01$) (Fig. 1a).

4.2. VCAM-1 immunoblots

After normalizing the density of VCAM-1 expression with beta-actin, it was observed that TNF-α significantly induces VCAM-1 ($p < 0.01$), which was further induced when cells were exposed to combined hypoxia + TNF-α ($p < 0.01$). Expression was suppressed when cells were exposed to aglycemia and given further stress with hypoxia, aglycemia and TNF-α (Fig. 1b).

4.3. ICAM-1 ELISA

TNF- α increased ICAM-1 ($P < 0.01$), and its expression was further increased with combined hypoxia + TNF-α ($p < 0.01$) and significant expression with hypoxia + aglycemia and TNF-α+hypoxia + aglycemia ($p < 0.01$) (Fig. 2a)

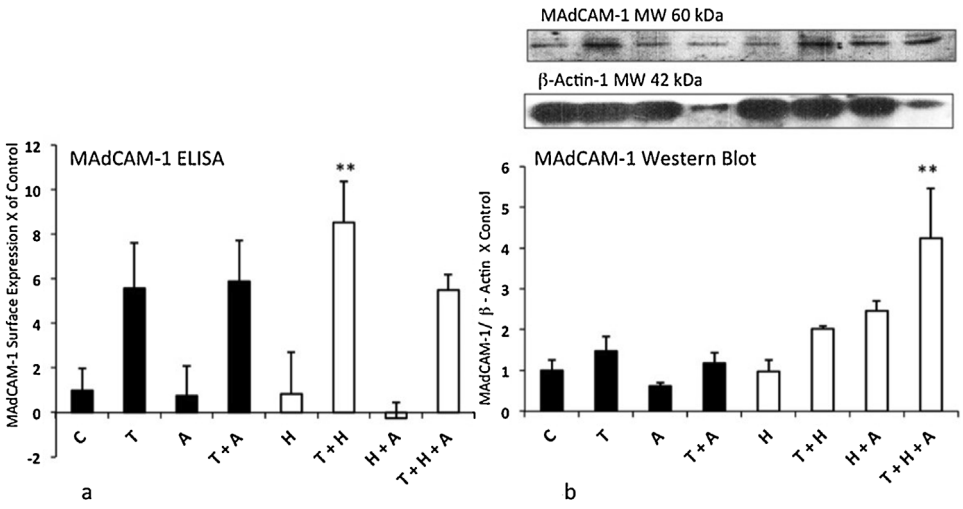

Fig. 3. a) Surface expression of MAdCAM-1 molecule under 8 different conditions including C = Control, T = TNF-α, A = Aglycemia, A + T = TNF-α and Aglycemia, H= Hypoxia, H + T = TNF-α and Hypoxia, H + A = hypoxia + aglycemia, T+ H + A = TNF-α and hypoxia + aglycemia. *n = 8, *p < 0.05, ** p < 0.01, *** p < 0.001.* b) Immunoblotting of MAdCAM-1 normalized with β-actin with 8 different treatment conditions including C = Control, T = TNF-α, A = Aglycemia, A + T = TNF-α and Aglycemia, H= Hypoxia, H + T = TNF-α and Hypoxia, H + A = hypoxia + aglycemia, T+ H + A = TNF-α and hypoxia + aglycemia. *n = 3, *p < 0.05, ** p < 0.01, *** p < 0.001.*

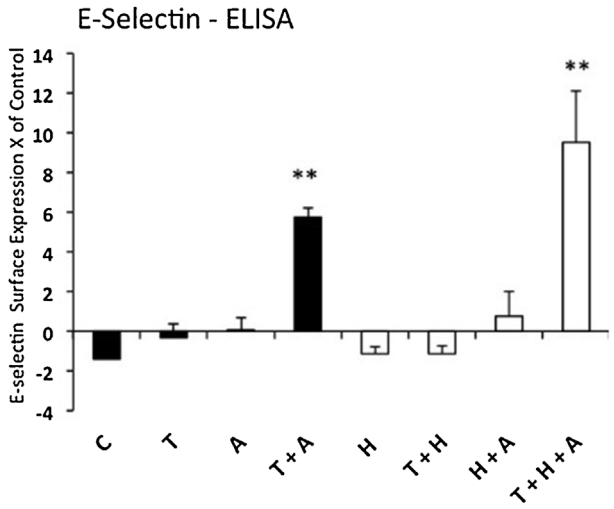

Fig. 4. Surface expression of E-selectin molecule under 8 different conditions including C = Control, T = TNF-α, A = Aglycemia, A + T = TNF-α and Aglycemia, H= Hypoxia, H + T = TNF-α and Hypoxia, H + A = hypoxia + aglycemia, T+ H + A = TNF-α and hypoxia + aglycemia. *n = 8, *p < 0.05, ** p < 0.01, *** p < 0.001.*

4.4. ICAM-1 immunoblots

After normalizing the data with the beta actin, ICAM-1 expression was not statistically significant, although there was an increasing trend with TNF-α, hypoxia + aglycemia, either alone or in combination with aglycemia (Fig. 2b).

4.5. MAdCAM-1 ELISA

Surface expression was significantly induced with hypoxia + TNF-α (<0.01), however with other conditions, such as TNF-α alone, combined aglycemia + TNF-α, and hypoxia + aglycemia + TNF-α up to 5X induction was noticed compared to control but it was not statistically significant (Fig. 3a).

4.6. MAdCAM-1 immunoblots

IMEC showed statistically significant expression of MAdCAM-1 only with combined hypoxia + aglycemia + TNF-α ($p < 0.01$) (Fig. 3b).

4.7. E-selectin ELISA

Increased surface expression of the E-selectin was detected on exposure to aglycemia + TNF-α (<0.01) and with hypoxia + aglycemia + TNF-α (<0.01) (Fig. 4). Other experimental conditions did not show significant difference.

4.8. E-selectin immunoblot

No expression was noticed with E-selectin expression in any of the experimental conditions (Data not shown).

4.9. IMEC metabolism

MTT assay showed significant suppression of cell metabolism with combined aglycemia + TNF-α ($p < 0.01$), which was further suppressed when cells were exposed to hypoxia + aglycemia + TNF-α ($p < 0.01$). Increase in cell metabolism occurred with other conditions i.e. TNF-α (p 0.05), hypoxia ($p < 0.01$), combined hypoxia + TNF-α ($p < 0.01$) and hypoxia + aglycemia (Fig. 5).

4.10. Cell detachment

Crystal violet staining showed that hypoxia + aglycemia plus TNF-α appeared to significantly ($p < 0.05$) stress cells, increasing their detachment (Fig. 6).

5. Discussion

Several theories have been proposed for the pathogenesis of gut inflammation and injury in NEC. [1–4] Studies have suggested that an ischemic reperfusion injury exacerbates the inflammatory processes seen in patients with NEC [2,3] and may result from intracellular metabolic alterations leading to further cell injury and death. Although hypoxia may occasionally trigger gut inflammation and/or injury, other factors such as prematurity, hemodynamic

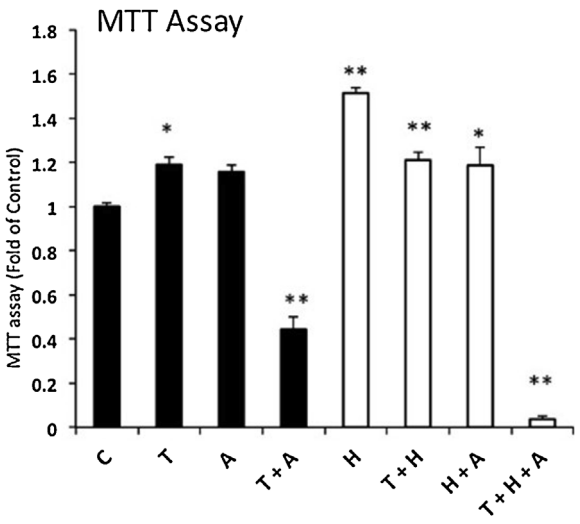

Fig. 5. MTT assay showing the metabolic status of the endothelial cells under 8 different conditions including C = Control, T = TNF-α, A = Aglycemia, A + T = TNF-α and Aglycemia, H= Hypoxia, H + T = TNF-α and Hypoxia, H + A = hypoxia + aglycemia, T+ H + A = TNF-α and hypoxia + aglycemia. *n = 12, *p < 0.05, ** p < 0.01, *** p < 0.001.*

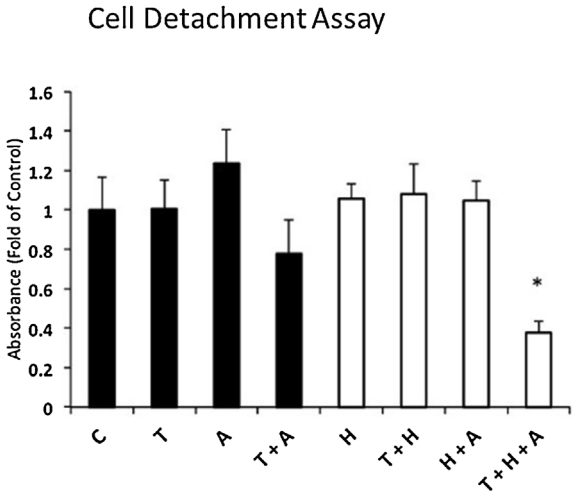

Fig. 6. Colorimetric cell adhesion using crystal violet staining technique under 8 different conditions including C = Control, T = TNF-α, A = Aglycemia, A + T = TNF-α and Aglycemia, H= Hypoxia, H + T = TNF-α and Hypoxia, H + A = hypoxia + aglycemia, T+ H + A = TNF-α and hypoxia + aglycemia. *n = 12, *p < 0.05, ** p < 0.01, *** p < 0.001.*

instability, gut dysmotility, altered gut flora, aggressive feeding practices have a role in initiating gut inflammation and injury [1–4]. These factors may affect cellular nutrition and hypoxia [2,4]. Our studies suggest that cellular metabolic stress with glucose depletion and/or hypoxia is related with inflammation.

Nowicki et al. suggested that ischemic-reperfusion injury to the gut is age dependent, with studies in neonatal pigs showing a greater risk for endothelial injury during the first 3 days of life which was also associated with poorer outcomes, compared to 35 day neonates. Endothelial cells play a major role in pathological processes in NEC controlling blood flow, nutrient delivery, metabolic hemostasis, immune trafficking and inflammation with activated capillary endothelial cells exhibiting leakage, enhanced leukocyte binding, trans-endothelial migration, procoagulant activity and angiogenesis [20].

Upon inflammatory activation, leukocytes roll on endothelium using P- and E-selectins [21–24] prior to firm adhesion on VCAM-1 and ICAM-1 which are also strongly expressed on activated endothelial cells. MAdCAM-1 is expressed on a subset of endothelium in Peyer's patches of small intestines, but also splenic sinusoids and mesenteric vessels. Together these ECAMs recruit innate and adaptive immune cells, and can also intensify inflammatory responses leading to host injury.

Under non-stimulated conditions, we saw low expression of VCAM-1, ICAM-1 and MAdCAM-1. E-selectin expression was not normally observed, but was expressed after TNF-α+aglycemia or TNF-α+ hypoxia + aglycemia exposure, suggesting that it is expressed under inflammatory and injury processes. We found an approximately 2X increase (Fig. 1a) in VCAM-1 surface expression compared to control cells exposed to TNF-α or a combined hypoxia + TNF-α challenge. We found that hypoxia + aglycemia + TNF-α strongly depressed endothelial metabolism (Fig. 5) but these cells expressed VCAM-1 and presented adhesive this determinant consistent with leukocyte recruitment. Immunoblot analysis showed a significant increase in the VCAM-1 following TNF-α and with hypoxia + TNF-α (Fig. 1b). Under normal conditions, ICAM-1 was expressed at low levels in the endothelium, but was up-regulated with TNF-α, TNF-α+hypoxia, hypoxia + aglycemia and TNF-α+hypoxia + aglycemia (Fig. 2a). Immunoblot assays of ICAM-1 showed that endothelial cells reduced the expression of ICAM-1 under all the experimental conditions except with hypoxia + aglycemia, and with hypoxia + aglycemia + TNF-α, where the expression of ICAM-1 within these cells was constant (Fig. 2b).

MAdCAM-1 surface expression increased 6 fold with aglycemia + TNF-α and TNF-α compared to control alone and 8-fold more with hypoxia (Fig. 3a). Immunoblotting revealed upregulation of MAdCAM-1 when TNF-α hypoxia and aglycemic challenges were combined (Fig. 3b). E-selectin was up-regulated with combined aglycemia + TNF-α challenge and with hypoxia + aglycemia + TNF-α exposures (Fig. 4); however, immunoblot failed to show expression under these treatment conditions, however, this particular antibody clone, 10E9.6 has not been used in western blotting but is active in blocking and ELISA studies (http://www.bdbiosciences.com/ptProduct.jsp?ccn=553748).

Haraldsen et al. reported disparity of E-selectin in HIMEC/HUVEC with E-selectin peaks at 4–6 hrs thereafter returning to baseline by 24 h in response to TNF-α [25]. Since we only detected E-selectin in the cell ELISA assay but not western blotting, our data on E-selectin suggests it is functionally active at the cell surface of IMEC (at least those derived from H-2 K(b)tsA58 mice), but so far we cannot determine its' cytoplasmic expression levels.

TNF-α increased expression of several ECAMs that govern both innate and adaptive immunity. The results from these experiments suggest that under combined stressful conditions such as exposure to hypoxia plus aglycemia with TNF- α, cell metabolism (Fig. 5) and binding to the underlying matrix (Fig. 6) were suppressed, but these endothelial cells strongly expressed multiple adhesion molecules. Cell metabolism was increased under hypoxic conditions compared except in the case of a combination of hypoxia plus aglycemia and TNF-α exposure.

Under non-metabolically challenged conditions, endothelial induction of adhesion molecules in response to challenge with inflammatory cytokines e.g. TNF-a is mediated by NF-kB, tyrosine kinases, p42/44, p38 MAP kinases, poly-ADP ribose polymerase (PARP) pathways as well as proteasome activity [26]. Since all of these pathways can be enhanced by the presence of reactive oxygen species, particularly following metabolic challenge, our findings here are consistent with metabolic enhancement of adhesion molecules in endothelial cells which may represent a relative super-stimulus compared to activation by cytokines e.g. TNF-a alone.

Our cell ELISA studies showed that under several metabolic challenges, there were increases in the expression of several adhe-

sion molecules; these increases in apparent abundance did not consistently show parallel changes in total protein expression by immunoblot, which may be consistent with mobilization of these ECAMs to the cell surface, without necessarily inducing novel protein expression of these ECAMs.

Although there are some limitations in this in vitro culture models, our data suggest that endothelial cells may exhibit synergistic activation in the face of combined metabolic and cytokine challenges in the NEC-inflamed tissue environment which aggravate this conditions. Apparently, some metabolic stresses lead to exposure of pre-existing pools of ECAMs which may support inappropriately high levels of leukocyte binding and leukocyte dependent injury. Further evaluation and characterization of inflammatory markers in an in vivo model in the necrotic gut tissue is needed to enhance our understanding of the endothelial dysfunction mechanisms in NEC gut injury.

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