

ORIGINAL**The oxidative stress of hyperglycemia and the inflammatory process in endothelial cells**

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Abstract : Hyperglycemia and insulin resistance are common in many critically ill patients. Hyperglycemia increases the production of reactive oxygen species in cells, stimulates the production of the potent proinflammatory cytokines IL-8 and TNF-alpha, and enhances the expression of haem oxygenase-1, an inducible stress protein. It has been shown that administration of insulin and the semi-essential amino acid glutamine have been beneficial to the septic patient. The aim of our study is to test whether these two molecules, glutamine and insulin used in combination attenuate the proinflammatory responses in endothelial cells which have been triggered by hyperglycaemia. Our results demonstrate that a combination of insulin and glutamine are significantly more effective in reducing the expression of IL-8, TNF-alpha and HO-1 than insulin or glutamine alone.

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INTRODUCTION

Sepsis and its complications continue to represent a significant cause of mortality among hospitalized patients. It includes a systemic inflammatory response to infection and when associated with organ dysfunction it is known as septic shock (1). Severe sepsis and septic shock are the primary causes of the multiple organ dysfunction syndrome (MODS) and one of the common causes of deaths (2). Many water soluble mediators with pro and anti-inflammatory actions such as interleukins (IL-6, IL-8, IL-10) and tumour necrosis factor alpha (TNF-alpha) appear to play a core role in sepsis. The disease conditions are still frequently encountered conditions in today's hospital environment despite the

growing armamentarium of antibiotics. This persistent high mortality rate is unacceptable as it is ranked above stroke and myocardial infarction. Many sepsis patients have at least one other dysfunction and deaths are often attributed to these conditions rather than to sepsis (3, 4). Possible reasons for this high mortality is late recognition and inappropriate treatment before entering the intensive care unit.

Sepsis is also a very complex disease state complicated by heterogeneous presentation. Hyperglycemia and insulin resistance are common in critically ill patients even without having had diabetes. Hyperglycemia or relative insulin deficiency or both during critical illness may directly or indirectly confer a predisposition to complications (5, 6) such as multiple organ failure and death. It has been shown that intensive insulin therapy to maintain the blood glucose at or below 110mg per deciliter reduces morbidity and mortality among critically ill patients (5).

Gram positive organisms, malarial parasite fungi

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and endotoxin containing organisms can trigger sepsis. Bacteremia or endotoxin can stimulate the innate immune system, endothelial cells and other cells (7). Hyperglycemia increases the production of reactive oxygen species inside aortic endothelial cells and is known to be proinflammatory. It is likely that glucose stimulates the production of TNF-alpha, a potent proinflammatory molecule, which is capable of activating NADPH dependent oxidase, enhances nuclear factor kappa B (NFκB) and intracellular cell adhesion molecule-1 (ICAM-1) expression. The stimulated cells release interleukins and TNF-alpha which have profound effects on the cardiovascular system, kidneys, liver, central nervous system and coagulation cascade (8). It is reported that interleukin-8 is the most potent one that is rapidly produced in the lung well before the recruitment of the polymorphonuclear leucocytes. The use of enteral glutamine supplementation in critical illness is well established. Although there was no statistical differences in mortality between the glutamine supplemented versus non-supplemented patients, there was evidence that glutamine reduced the incidence of pneumonia, sepsis and bacteremia (9-12). It has been shown that glutamine therapy caused attenuation of the inflammatory interleukins and enhanced expression of heat shock proteins (13). Since heme oxygenase-1 (HO-1) is an inducible stress protein, which confers cytoprotection against oxidative stress *in vitro* and *in vivo* (14), we also included the measurement of HO-1 in our model. Our aim is to establish an *in vitro* model with the human umbilical cord endothelial cells and test whether glutamine and insulin used individually or together are cytoprotective in reducing proinflammatory responses in cells which have been triggered by hyperglycaemia.

MATERIALS AND METHODS

Cell culture

Endothelial cells were obtained from Vec Technologies Inc, New York. Frozen cells were unpacked immediately and stored in liquid nitrogen until ready to use. When ready for culture the vials were thawed at 37°C. When thawed the cells were transferred into T-25 flask coated with 50µg/ml of fibronectin and the cells were immersed in 5 ml of complete medium (MCDB-B-1). The cells were incubated at 37°C and 5% CO₂. After 12 to 18 hrs when the cells were attached, the media were

changed every two hrs until the cells reached confluency. At confluence, the cells were trypsinised, counted and aliquoted into different vials and frozen.

Cell treatment

The frozen vial was removed and passaged. When the flask reached confluency, the cells were trypsinised and counted. 10⁵ cells were added to each flask and different concentrations of glucose ranging from normal values (5.6 mM) to hyperglycemic levels (10 mM and 20 mM) were added to each individual flask. The hyperglycemic flasks (glucose concentration 20 mM) were divided into 3 groups. In the first group different concentrations (40 mM) of glutamine were added. In the second group, insulin (1.0 × 10⁻¹², 1 × 10⁻⁹, 1 × 10⁻⁶ units/ml) were added alone. In the third group glutamine and insulin were added and the flasks were incubated for the required length of time (7 days). Cells were harvested and frozen until required for analysis of TNF-alpha and HO-1 while culture media was retained for IL-8 analysis.

Measurement of TNF-alpha and HO-1 expression

Frozen cells were thawed and resuspended in 200 µl PBS. They were then lysed by using Lysis Buffer and vortexed for 15 s. Total mRNA was extracted using Roche High Pure RNA isolation kit (Roche Diagnostics, Mannheim). Messenger RNA (mRNA) was then converted to complementary DNA (cDNA). The cDNA was converted to double stranded DNA using Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics, Mannheim) and TNF-alpha and HO-1 specific sense primer. TNF-alpha and HO-1 cDNA were then amplified by TNF-alpha specific primer set (TNF-alpha-F: 5' cag agg gcc tgt acc tca tc ; TNF-alpha-R: 5' gga aga ccc ctc cca gat ag) and HO-1 primer set (HO-1-F: 5' tct ctt ggc tgg ctt cct ta ; HO-1-R: 5' ggc cgt gtc aac aag gat ac) respectively using Fast Start DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim) and quantified using Roche LightCycler (15). The formula (16) used for calculation of the relative quantity is as follows: Ratio = (E_{target})^{ΔC_Ptarget(control-sample)} / (E_{ref})^{ΔC_Pref(control-sample)}

Measurement of IL-8

Cytokine concentrations in the cell culture media were determined using sandwich ELISA for IL-8 (17). Briefly, 96-well immunomaxisorb plates (Nunc, Roskilde) were coated overnight at 4°C with cytokine specific mAb. TNF-alpha in the su-

pernatant was detected with polyclonal rabbit anti-human TNF-alpha followed by peroxidase conjugated, goat anti-rabbit IgG (Jackson Immuno-research Laboratories, Westgrove). IL-8 was detected by biotinylated polyclonal rabbit anti-human IL-8 followed by peroxidase-conjugated streptavidin. Tetramethylbenzidine was used as a substrate for peroxidase and spectrophotometry was performed at 450 nm.

RESULTS

Under hyperglycemic conditions (20 mM) the expression of IL-8 was 1.2 times higher than under normal glucose levels. In hyperglycemic conditions, when the cells were treated with 40 mM of glutamine or insulin, the IL-8 level was not reduced significantly ($p > 0.05$). In hyperglycemic cells treated with the highest concentration of insulin (10^{-6} M) and glutamine (40 mM) together, the IL-8 level was reduced by 2 times, $p < 0.001$ (Fig. 1). Under

the same hyperglycemic conditions the expression of HO-1 was 2.7 times higher than under normal glucose levels (Fig. 2). This is in agreement with the fact that HO-1 is an inducible stress protein. This experiment clearly demonstrates that HO-1 is largely produced under stress of hyperglycaemia. In hyperglycemic cells treated with 40 mM of glutamine or 10^{-6} M insulin, the HO-1 level was reduced 3.2 times and 6.4 times respectively. In the cells treated with insulin and glutamine together the HO-1 level was reduced 5.1 times even at the lowest concentration of either insulin or glutamine. Under the same conditions, the expression of TNF-alpha was 3 times higher than under normal glucose level (Fig. 3). In hyperglycemic cells treated with 40 mM of glutamine or 10^{-6} M insulin, the TNF-alpha level was reduced about 5.7 times and 6.3 times respectively. In hyperglycemic cells treated with insulin and glutamine together, the TNF-alpha level was reduced 3.8 times at the lowest concentration of either glutamine or insulin.

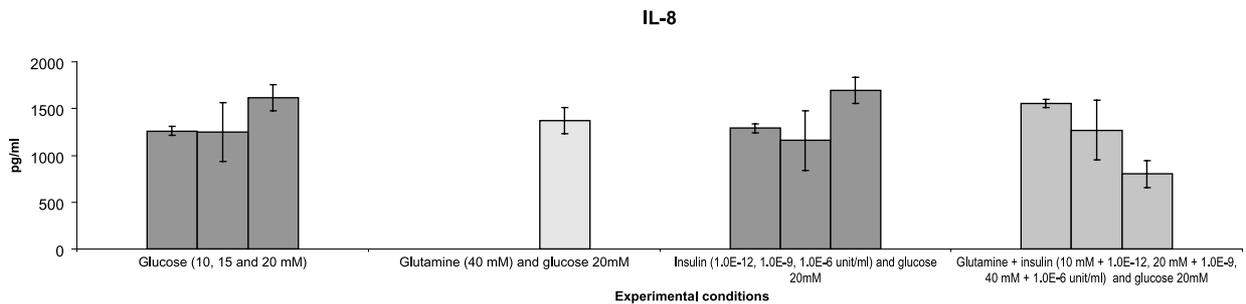


Fig. 1. Determination of endothelial IL-8 levels under different treatment conditions. IL-8 levels were measured by sandwich ELISA.

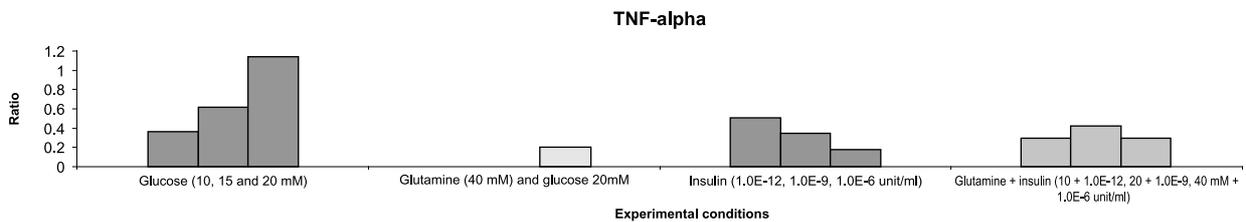


Fig. 2. Determination of endothelial TNF-alpha expression under different treatment conditions. Values are means of triplicate experiments. Expression was measured by real-time PCR.

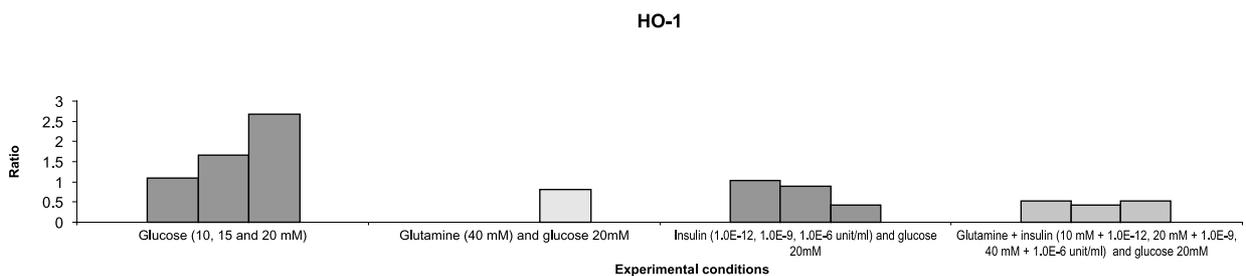


Fig. 3. Determination of endothelial HO-1 expression under different treatment conditions. Values are means of triplicate experiments. Expression was measured by real-time PCR.

DISCUSSION

It has been hypothesized that hyperglycemia or relative insulin deficiency or both during critical illness may directly or indirectly confer a predisposition to complications (18, 19) such as severe infections, polyneuropathy, multiple organ failure and death. It has been shown that intensive insulin therapy to maintain the blood glucose at or below 110 mg/dl reduces morbidity and mortality among critically ill patients (18). Gram positive organisms, malarial parasites, fungi and endotoxin containing organisms can trigger sepsis. The invading organisms proliferate and produce bacteremia or may release endotoxin that stimulates the innate immune system, endothelial cells and other cells (7). These stimulated cells release proinflammatory interleukins like IL-8, TNF-alpha, endorphins, nitric oxide, and migration inhibitory factor (MIF) which in turn augment the expression of various adhesion molecules.

In order to test this hypothesis and the mechanism involved in the predisposition to complications under hyperglycemic conditions we set up an *in vitro* model with endothelial cells. The endothelial cells were subjected to hyperglycemic conditions to test the effect hyperglycemia on the endothelial cells with respect to the production of IL-8, TNF-alpha and HO-1.

It is known that hyperglycemia increases the production of reactive oxygen species in aortic endothelial cells and is known to be proinflammatory. What could be the mechanisms by which glucose stimulate proinflammatory events? It is likely that glucose stimulates the production of TNF-alpha, a potent proinflammatory molecule which is capable of activating the NADPH dependent oxidase, enhances NFκB activity and ICAM-1 expression (20). This ability of glucose to stimulate TNF-α production, formation of free radicals, the expression of adhesion molecules and suppression of nitric oxide (NO) generation could be responsible for increased morbidity, increased mortality and the high incidence of sepsis and septic shock in diabetes mellitus. In contrast to glucose, insulin suppresses NFκB expression, free radical generation especially superoxide, MIF production and enhances NO generation and inhibits inflammatory processes (21). Furthermore, it has been shown that advanced glycation endproducts produced during oxidative stress

of hyperglycaemia upregulate expression of vascular endothelial growth factor, tissue factor and the proinflammatory cytokines TNF-alpha and IL-8 according to length of stimulation.

A tight control of hyperglycemia with insulin and the parenteral administration of the semi-essential amino acid, glutamine have been shown to improve the outcome of critically ill patients (22). Our results demonstrate that when endothelial cells were made hyperglycemic, they expressed a large amount of the heat shock protein HO-1 which was reduced when cells were treated with insulin or a combination of insulin and glutamine. Our results shows that at the cellular level insulin and glutamine attenuated the expression of inflammatory cytokines, such as TNF-alpha and IL-8, and reduced the oxidative stress of hyperglycemia. Along with it, our results also demonstrate that when endothelial cells are treated with both insulin and glutamine together, the reduction in the inflammatory process was far more effective than when treated with either of them alone. Thus, when used in combination insulin and glutamine may be therapeutically beneficial for patients who present with sepsis.

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