Hyperglycemia, oxidative and nitrosative stress affect antiviral, inflammatory and apoptotic signaling of cultured thymocytes

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A high prevalence of various infectious diseases is reported in diabetic patients, which may suggest impaired innate immunity against different pathogen-associated molecular patterns. This study investigated the effects of hyperglycemia, oxidative stress (H_2O_2), nitric oxide (NO) and peroxynitrite (ONOO⁻) on the modulation of antiviral (MDA-5, IRF-3 and phospho-IRF-3), inflammatory (NF- κ B) and pro/anti-apoptotic molecules (Bax and Bcl-2) in BALB/c mice thymocytes. Each of the experimental conditions, except the weakest NO concentration, resulted in down-regulation of MDA-5, IRF-3 and phospho-IRF-3. In contrast, each of the experimental conditions elicited up-regulation of NF- κ B, Bcl-2 and Bax. These results suggest that hyperglycemia, oxidative and nitrosative stress may contribute to the reduced immunity of the host by altering the MDA-5/IRF-3/phosphoIRF-3 axis, as well as contributing to the mechanisms of inflammatory reaction via increased NF- κ B, and to augmented turnover rate of thymocyte cells via Bcl2/Bax up-regulation.

Keywords: oxidative stress, peroxynitrite, glucose, IRF-3, phospho-IRF-3, MDA-5, NF- κ B, Bcl-2, Bax, thymocyte

Introduction

Patients with metabolic decompensation of diabetes mellitus are at higher risk of certain invasive infections, especially in the postoperative period and during hemodialysis.¹⁻³ A great number of systemic and local host factors in diabetes may contribute to greater susceptibility to viral infections. Both hyperglycemia and oxidative stress have been associated with immunological changes.⁴⁻⁶ In spite of good clinical experience, the exact downstream signaling pathway alterations, due to poor metabolic

control of diabetes, have not been completely explored. Chronic hyperglycemia primes the innate immune system for an exaggerated inflammatory response, followed by marked enhancements in cytokine and chemokine release, increased leukocyte activation, superoxide ($O_2^{\bullet-}$) release and nitric oxide (NO[•]) production.⁷ Once formed in higher concentrations, NO is considered as an active agent in several pathologies based on inflammation and plays an important role in atherosclerosis. Increased production of reactive oxygen species (ROS) also contributes to the inflammatory reaction. Once formed, superoxide anion radical ($O_2^{\bullet-}$) reacts rapidly with NO[•], producing the extremely reactive peroxynitrite (ONOO⁻) which mediates oxidation, nitrosation, and nitration reactions.⁸⁻¹⁰

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The innate immune responses to viral infections represent the multistep processes requiring the recognition of virus infection, activation of multiple signal transduction cascades and the transcription of specific antiviral gene products. The innate immune system recognizes viral nucleic acids, comprising viral genomes or generated during viral replication, by two different classes of pathogen-associated molecular patterns (PAMPs): the Toll-like receptors (TLR3, TLR4, TLR7, and TLR9), capable of detecting endosome phagocytosed nucleic acid products, or the helicase systems, known as the retinoic acid-induced protein 1 (RIG-I) and melanoma differentiation-associated protein-5 (MDA-5), capable of 'sensing' cytoplasmic dsRNA generated during viral replication. MDA-5-deficient animals exhibit a selectively impaired antiviral response to picornavirus, a main class pointed out as a possible cause of diabetes.¹¹ Viral RNA binding by MDA-5 activates signaling via the interferon promoter stimulator 1 (IPS-1), further activating the IRF-3 kinases TBK1 and IKK ϵ . These pathways confer signaling to the latent cytoplasmic transcription factor IFN regulatory factor-3 (IRF-3) and nuclear factor- κ B (NF- κ B). Activated IRF-3, together with NF- κ B and AP-1, accumulate in the nucleus, bind to the IFN- β promoter, and stimulate IFN-I transcription. Functional IRF-3, as well as signal-induced co-ordination of NF- κ B and IRF-3, is absolutely required for the optimally controlled host antiviral immune response.^{12,13} Understanding the mechanisms by which IRF-3 and MDA-5 may be down-regulated provides insight into how the host response via the immune system may be blocked and how viruses may replicate successfully in inflammatory or hyperglycemic conditions.^{12,14} NF- κ B has been implicated in the regulation of gene transcription that contributes to cytokine generation, lymphocyte maturation, protection from TNF- α -induced apoptosis and antigen processing and presentation by MHC class I molecules. It has proven to play an essential role in the generation of an acute inflammatory response to different infections, and is also implicated in chronic inflammatory diseases.^{15,16}

The effects of a hyperglycemic milieu and metabolic stress parameters on vulnerability to viral infections in diabetic conditions have not been fully explored. In our study, the possible impact of hyperglycemia, oxidative stress (H₂O₂), nitric oxide (NO) and peroxynitrite (ONOO⁻) on antiviral (MDA-5 and IRF-3), inflammatory (NF- κ B) and pro/apoptotic molecules (Bax and Bcl-2) modulation on BALB/c mice thymocytes was evaluated.

Materials and methods

Preparation of thymocyte cell culture

The thymocyte cell culture preparation was described in our previous report.¹⁷ Briefly, the thymus of BALB/c mice was extirpated using sterile technique and placed in RPMI-1640/10% fetal calf serum (FCS). Thymocytes were released by teasing the thymus through a steel-mesh. After removal of stroma, the cells were washed twice with RPMI-1640/10% FCS. Culture medium (RPMI-1640) contained 25 mM HEPES, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μ g/ml) and 10% FCS. In order to mimic metabolic decompensation of diabetes mellitus, thymocyte cultures were divided into 12 groups (each comprising eight samples): I, hyperglycemia (10, 50 and 100 mmol/l glucose); II, oxidative stress (0.1, 1 and 10 mmol/l hydrogen peroxide); III, NO production (0.1, 1.0 and 10 mmol/l sodium nitroprusside); and IV peroxynitrite (ONOO⁻) formation (0.01, 0.1 and 0.5 mmol/l authentic peroxynitrite). Authentic peroxynitrite was synthesized by quench-flow technique, explained in our previous report.¹⁸ The control thymocyte group was incubated in RPMI-1640/10% FCS medium. Each sample contained 100 μ l of cells + 800 μ l of medium + 100 μ l of test condition (medium alone for controls). The cells were incubated for a period of 6 h (3–6 h was referred to as the optimal time for transcription factors up/down-regulation) at 37°C in a standard CO₂ incubator. After the proposed period, the cells were centrifuged, the supernatant discarded and the remaining cells dissolved in 200 μ l of PBS.

Determination of MDA-5, IRF-3, phospho-IRF-3, NF- κ B, Bcl-2 and Bax

Aliquots of 50 μ l of each cell suspension were transferred into 96-well U-bottom plates, (5 $\times$ 10⁵ cells/well), fixed by using 70% methanol and permeabilized with 1% Triton. For determination of MDA-5, IRF-3, phospho-IRF-3, NF- κ B, Bcl-2 and Bax, specific antibodies were used, purchased from Santa Cruz Biotechnology and Cell Signaling Technology: for MDA-5 (C-16: sc-48031), IRF-3 (FL-425: sc-9082), phospho-IRF-3 (Ser396 4D4G), NF- κ B (p65 (C-20): sc-372 epitope mapping at the C-terminus of NF- κ B p65), Bcl-2 (N-19: sc-492) and Bax (P-19: sc-526). The cells were incubated with the corresponding primary antibody, washed three times, and then incubated with the FITC-conjugated secondary antibody. The excess of antibody following staining was washed by adding PBS to each well. The mean fluorescence intensity (MFI; logarithmic scale) of cell populations was determined, and the results

Figure 1 (A) MDA-5, (B) IRF-3, (C) phospho-IRF-3, (D) NF- κ B, (E) Bcl-2 and (F) Bax expression under hyperglycemic, oxidative and nitrosative stress conditions. Freshly isolated thymocytes were divided into the following groups (each comprising 8 samples): I, hyperglycemia (10 and 50 and 100 mmol/l glucose); II, oxidative stress (0.1, 1 and 10 mmol/l hydrogen peroxide); III, NO production (0.1, 1 and 10 mmol/l sodium nitroprusside); and IV, peroxynitrite (ONOO⁻) formation (0.01, 0.1 and 0.5 mmol/l authentic peroxynitrite). The results were compared to control samples incubated in RPMI-1640 medium supplemented with the 10% FCS only

presented are obtained following subtraction of the fluorescence intensity of secondary antibodies alone. The values were analyzed on a Victor Perkin Elmer–Wallace multiplate reader.

Determination of lipid peroxidation (MDA level)

The level of lipid peroxidation was assayed by thiobarbituric acid reactive substances assay (TBARS), in the remaining cell suspension. The cell

suspensions were heated with TBA and the pink chromogen was quantified by spectrophotometry at 532 nm according to the method of Ohkava *et al.*¹⁹

Statistical analysis

The results are presented as mean $\pm$ SD. The statistical significance was evaluated using ANOVA. The level of significance for all analyses was defined as $p = 0.05$.

Results

Each of the experimental conditions, except the lowest concentration of NO, were able to down-regulate MDA-5, IRF-3 and phospho-IRF-3, important components of the surveillance and cellular innate immunity response (Fig. 1A–C). Concerning NF- κ B, Bcl-2 and Bax, thymocyte cultures responded to experimental conditions in an opposite way. Each of the experimental conditions used, elicited NF- κ B up-regulation (Fig. 1D). Each treatment promoted the up-regulation of Bcl-2 significantly (Fig. 1E). Since oxidative and hyperglycemic conditions are often characterized as initiating apoptosis, the question arises whether they may change Bax levels despite Bcl-2 up-regulation. We determined that each of the experimental conditions used was capable of evoking early response via Bax up-regulation as well (Fig. 1F). The lipid peroxidation (MDA) level increased significantly during oxidative and nitrosative stress conditions (Fig. 2), while NO exerted significant decrease in MDA level. Glucose did not change the MDA level significantly.

Discussion

Different types of infection have been shown to cause considerable morbidity and mortality in patients with diabetes. They have an influence on metabolic control because of activation of the immuno-inflammatory cascade and acceleration of atherosclerosis.^{1,5} Conversely, metabolic dysregulation of diabetes may facilitate infection. Stress hyperglycemia frequently occurs in critically ill patients. It has been observed that intensive insulin treatment, aimed at maintaining normal blood glucose concentration in critically ill patients, significantly reduces the incidence of severe infections and related morbidity and lethal multiple organ failure by improving the innate immune response.²⁰ The results obtained in our study suggest that metabolic stress parameters may play a pivotal role in dysregulation of the antiviral and pro-inflammatory response (Fig.

Figure 2 Lipid peroxidation (MDA level) under hyperglycemic, oxidative and nitrosative stress conditions. Freshly isolated thymocytes were divided into the following groups (each comprising 8 samples): I, hyperglycemia (10 and 50 and 100 mmol/l glucose); II, oxidative stress (0.1, 1 and 10 mmol/l hydrogen peroxide); III, NO production (0.1, 1 and 10 mmol/l sodium nitroprusside); and IV, peroxynitrite (ONOO⁻) formation (0.01, 0.1 and 0.5 mmol/l authentic peroxynitrite). The results were compared to control samples incubated in RPMI-1640 medium supplemented with the 10% FCS only

1A–E). The ability of our experimental conditions to down-regulate the MDA-5 (Fig. 1A), IRF-3 (Fig. 1B) and phosphoIRF-3 (Fig. 1C) may have a great impact in controlled activation of the antiviral response. IRF-3 has important roles in host defense against viral infection, transcriptional induction of type I interferons (IFNs), expression of IFN-responsive genes, development of the immune system and apoptosis.^{13,21,22} In unstimulated cells, IRF-3 is present in the cytoplasm and its activation occurs via phosphorylation of C-terminal serine and threonine residue clusters, which induces its translocation to the nucleus, activating the DNA-promoter binding site. The activation usually occurs during viral infections. It was recently documented that the level of IRF-3 is susceptible to oxidative stress.²³ A role in immune cell growth control, differentiation and apoptosis has also been attributed to MDA-5.^{12,24} An altered MDA-5/IRF-3 axis is central in viral evasion of picornavirus and paramyxoviruses. The MDA-5/IRF-3 axis may be a subject of down-regulation especially during some viral infections, such as the inhibition of the IFN response of the severe acute respiratory syndrome (SARS) coronavirus (CoV) infection. In this way, the down-regulation of IRF-3-dependent innate antiviral

defenses may contribute to the establishment of SARS-CoV infection.²⁵ Besides their role in antiviral host defense, it has been documented that some IFN-stimulated genes associated with NF- κ B and IRF-3 activation are induced by Gram-negative bacteria lipopolysaccharide (LPS) and some bacterial species.^{26,27} Reactive oxygen species (ROS) play a central role in vascular damage and development of atherogenesis in diabetes,^{8,9,28,29} but their effect on the antiviral immune response has not yet been fully explored. The significant down-regulation that we observed in each of our experimental culture conditions may suggest that the antiviral response is compromised significantly when the metabolic control of diabetes is altered due to hyperglycemia, oxidative or nitrosative stress. In contrast, lower concentrations of NO may have an activatory effect on the host antiviral response (Fig. 1C). Downstream products of NF- κ B activation include inflammatory cytokines, tumor necrosis factor (TNF) and nitric oxide synthase (NOS) activation.¹⁵ In unstimulated cells, NF- κ B is found as an inactive heterodimer of p50/p65 subunits, bound to the NF- κ B inhibitor protein, I κ B. Upon stimulation, I κ B is phosphorylated and directed to the ubiquitin degradation pathway.¹⁶ NF- κ B is well known as a 'redox-sensitive' transcription factor, because ROS trigger a change in the cell that results in phosphorylation of the I κ B subunit.^{15,16} The NADPH oxidase enzyme system is one of the most important sources of superoxide in vascular cells. Regulation of NADPH oxidase by NF- κ B may explain how pro-inflammatory factors induce oxidative stress in atherosclerosis, hypertension, diabetes, stroke or heart failure. High glucose conditions have been shown to induce NF- κ B activation in a culture of vascular smooth muscle cells.³⁰ Studies of altered NO regulation and secretion, due to the local induction of 'inducible' NOS-2, suggested that it may have an impact on development and progression of atherosclerosis and diabetic complications. Nitrosative stress is of particular interest to endothelial damage in diabetes and the presence of nitrotyrosine in plasma is a reliable marker for development of diabetic complications.^{9,28} The effect of hyperglycemic conditions, as well as other factors related to the metabolic control used in our study, may suggest that each is capable of orchestrating the up-regulation of NF- κ B, triggering the inflammatory cascade. NF- κ B was activated in response to various experimental stimuli (Fig. 1D), but the highest concentrations of NO and ONOO⁻ did not exert the expected rise. A possible reason may be the decreased oxidative stress conditions, since the level of lipid peroxidation was

also reduced. Another explanation may be the tyrosine nitration of p65 subunit.³¹

Our data concerning the Bcl-2/Bax regulation (Fig. 1E,F) suggest that mitochondria may function as a cellular checkpoint under conditions of hyperglycemia, oxidative and nitrosative stress. Constitutive Bcl-2 up-regulation does not only prevent the infected cells from undergoing apoptosis, but renders the cells persistently infected. In this way, Bcl-2 may act as an anti-apoptotic, but not antiviral, protein.³² Since the apoptotic deletion of potentially autoreactive T-cells is involved in the maintenance of self-tolerance, the defective apoptotic cell clearance may facilitate the inappropriate presentation of potential autoantigens by the thymus antigen-presenting cells and may have an impact on the autoimmune response.^{32,33} Bcl-2 expression in endothelial cells reduces the increase in intracellular AGEs induced by higher glucose concentrations.³⁴ The results obtained in our study may be explained as an early compensatory response. It has also been demonstrated that Bcl-2, as an anti-apoptotic protein, is capable of blocking the lipid peroxidation and cumulative effects of oxidative stress which occur with apoptotic cell death in lymphocyte cultures.³⁵ Its potential ability to counteract the detrimental effects of cell damage caused by free radicals, may be explained by its antioxidative properties.³⁴⁻³⁷ It was proposed that Bcl-2 may function as the non-reactive trap system of AGE-producing free radical species, or may possess peroxidase or reductase activity, but such activities have not yet been detected.³⁸ Oxidative signals and NO trigger the accumulation of Bcl-2 in other cell types. NF- κ B has been shown to have an impact on increased Bcl-2 expression as well.³⁸ In the current study, Bax up-regulation was also observed (Fig. 1F). Since considerations for the anti-apoptotic Bcl-2 action include, besides its antioxidant ability, the interaction and neutralization of pro-apoptotic proteins such as Bax, and inhibition of the mitochondrial permeability transition that participates in promoting apoptosis, our results may again evoke Bcl-2 up-regulation as an early compensatory response to Bax cytotoxic stimulation. During autoimmune diabetes development, a number of T-cells was registered to be apoptotic. Although many T-cells became apoptotic, they were able to orchestrate inflammation and cytotoxicity at a level sufficient to induce β -cell death as demonstrated elsewhere.³⁹

Quantification of lipid peroxidation is essential to assess the role of oxidative injury in pathophysiological disorders. Lipid hydroperoxides represent the intermediates derived from unsaturated fatty acids, phospholipids, glycolipids, cholesterol

esters and cholesterol. Lipid peroxidation represents one of the major outcomes of free radical-mediated injury to tissue and may be used as a reliable marker of oxidative stress conditions. Peroxidation of lipids can greatly alter the physicochemical properties of membrane lipid bilayers, resulting in severe cellular dysfunction, among them the alteration of many redox-sensitive signaling molecules.^{23,40}

Conclusions

These results may suggest that hyperglycemia, oxidative and nitrosative stress may contribute to the mechanisms of inflammatory reaction, and/or to the inability of the host to rid itself completely of the virus via altered MDA-5/IRF-3/phospho-IRF-3 axis and NF- κ B regulation. New experimental systems would be aimed at addressing the question of whether modulating the expression of these molecules may lead to the design of future therapeutic regimens for the amelioration of the antiviral response in diabetic conditions.

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