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Glucose-modulated Tyrosine Nitration in Beta-Cells: Targets and Consequences

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Abstract

Hyperglycemia, key factor of the pre-diabetic and diabetic pathology, is associated with cellular oxidative stress that promotes oxidative protein modifications. We report that protein nitration is responsive to changes in glucose concentrations in islets of Langerhans and insulinoma beta-cells. Alterations in the extent of tyrosine nitration as well as the cellular nitroproteome profile correlated tightly with changing glucose concentrations. The target proteins we identified function in protein folding, energy metabolism, antioxidant capacity, and membrane permeability. Nitration of heat shock protein 60 in vitro was found to decrease its ATP hydrolysis and interaction with proinsulin, suggesting a mechanism by which protein nitration could diminish insulin secretion. This was supported by our finding of a decrease in stimulated insulin secretion following glycolytic stress in cultured cells. Our results reveal that protein tyrosine nitration may be a previously unrecognized factor in beta-cell dysfunction and the pathogenesis of diabetes.

Keywords

beta cells; islets of Langerhans; nitric oxide; oxidative stress; protein tyrosine nitration; glucose; insulin; diabetes

1. Introduction

It is increasing becoming apparent that oxidative stress underlies some of the most common disease that we now face such as diabetes, cancer, sepsis, and neurological disorders such as Alzheimer's disease. Over the last few years, how we view oxidant species have changed from purely destructive molecules, to entities that play a role in physiological regulation and we now understand that only when dysregulation occurs do they instigate the disease process. Our challenge today is to understand how the multitude of different oxidant species contributes to their biological effects. As is often the case, we only begin to understand the normal role after we extrapolate back from the disease conditions. In this paper we demonstrate that small

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changes in glucose levels can lead to physiological changes that if unchecked can lead to disease.

Type 2 diabetes is increasing in prevalence worldwide [1,2]. The pre-diabetic symptom-free period is characterized by the progression from a normal glucose-stimulated insulin response, and therefore normal glucose tolerance, to an increasingly abnormal glucose tolerance along with insulin resistance [3,4]. Abnormal glucose tolerance comprises an impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT). This results in increasingly severe hyperglycemic episodes especially postprandially as β -cell function gradually deteriorates. However, acute hyperglycemia may also occur during physiological stress and shock states, as a result of hypermetabolism and insulin resistance due to stress hormones and proinflammatory cytokines, especially if preexisting latent defects in insulin secretion fail to compensate [5–8]. The metabolic effects of catecholamines to treat hypotension during shock states can contribute to this hyperglycemia [9]. Thus acute hyperglycemia may develop in critically ill patients and increase both morbidity and mortality [10,11].

Acute and chronic hyperglycemia as well as acute glucose fluctuations can cause glucose toxicity, an important pathological factor associated with oxidative stress in general and a rise in cellular superoxide production in particular [12–19]. Superoxide can contribute to the activation of NF- κ B and the expression of proinflammatory cytokines like interleukin-1 β (IL-1 β), a main cytokine in the pathogenesis of type 2 diabetes [18,20]. IL-1 β is a primary mediator of inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) generation by β -cells [21]. In the presence of both, NO and oxidants like superoxide, reactive nitrogen species (RNS) such as peroxynitrite can be formed. Recently, Pacher et. al. described how relatively small changes in superoxide and NO can result in dramatic changes in the generation of peroxynitrite [18]. These RNS augment oxidative protein modifications like protein tyrosine nitration through various mechanistic pathways [18,22,23].

The insulin-secreting pancreatic islet β -cells, whose dysfunction is central to the pathophysiology of diabetes, are exceptionally susceptible to this kind of oxidative stress [19,24,25]. A major contributor to this susceptibility appears to be their low enzymatic antioxidant capacity to detoxify excess superoxide and hydrogen peroxide. This includes the activity of Cu/Zn superoxide dismutase (SOD), mitochondrial Mn-SOD, catalase, and glutathione peroxidase [26,27]. An NO-dependent occurrence of protein tyrosine nitration in islets [28–30] is consistent with these findings, especially because hyperglycemia induces IL-1 β production, iNOS expression, and activation of neuronal NOS in pancreatic islets [18, 31,32]. Increased levels of nitrotyrosine have further been correlated with reduced glucose-stimulated insulin secretion (GSIS) in human type-2 diabetic islets [33]. Thus hyperglycemic oxidative protein modifications in β -cells may be a major contributor to a dysfunction or even cell death.

How oxidative stress triggered by glucose toxicity affects protein tyrosine nitration in β -cells has not been studied in detail. In the present study, we identified 15 proteins in rat insulinoma cells and rat islets of Langerhans that are nitrated and whose nitration changes under different hyperglycemic conditions. We further analyzed the effects of nitration on one of the target proteins, the mitochondrial heat shock protein 60 (HSP60). Our findings support a novel mechanism by which tyrosine nitration of specific target proteins can impair the stimulation of insulin secretion in β -cells.

2. Materials and Methods

2.1 Cell culture

Insulin-secreting rat insulinoma RIN-5F β -cells (ATCC, CRL-2058), a clone of the RIN-m insulinoma cell line, were grown in RPMI 1640 medium with 2 mM glutamine. The media was supplemented with 5 mM D-glucose (normoglycemic standard), 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin and changed every 24 hours. The cells were grown at 37°C under isobaric conditions (5% CO₂, 95% air) in humidified atmosphere using Corning CellBIND culture material.

2.2 Preparation and Culture of Islets

Islets of Langerhans were isolated from male Sprague-Dawley rats (250–300g) by collagenase digestion as previously described [34]. Islets were cultured overnight in complete CMRL-1066 containing 5.5 mM D-glucose, 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C under an atmosphere of 95% air and 5% CO₂ prior to experimentation. These isolates are capable of producing insulin in response to glucose [35].

2.3 Conditions of Elevated Glucose Levels

To simulate *in vivo* conditions of normal fasting glucose (NFG), which is now linked to plasma glucose levels of less than 5.2 mM, cells were constantly cultured at 5 mM glucose and media changed every 24 hours. As IFG is related to glucose levels between 5.6 and 6.9 mM and IGT is associated with a postprandial hyperglycemia marked by glucose levels of 7.8–11 mM [36,37], RIN-5F cells and islets of Langerhans were exposed to 6.5, 8, and 11 mM D-glucose in RPMI 1640 or CMRL-1066. The time of exposure was 24 hours or 12 hours with intermittent phases of 5 mM and 11 mM glucose for RIN-5F cells. The intermittent exposure was used to simulate physiological fluctuations in glucose levels according to the fact that postprandial blood glucose level regularly peaks approximately 30–120 min after the start of a meal [36]. Selective iNOS inhibition by 50 μ M L-N6-(1-iminoethyl)-lysine [38] was used to investigate the contribution of iNOS as specified for the individual experiments.

2.4 Differential Detergent Protein Extraction of RIN-5F Cells

After exposure to fluctuating glucose levels for 12 hours in RPMI 1640 (2 h 11 mM, 2 h 5 mM, 2 h 11 mM, 4 h 5 mM, and 2 h 11 mM) cells were sprayed off, pelleted and washed three times with PBS. To obtain the fraction of soluble cytosolic proteins the cells were incubated in buffer containing 0.25 M sucrose, 10 mM HEPES, 1 mM EDTA, and 0.01% digitonin for 5 min on ice in the presence of protease inhibitors (5 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, and 24 μ g/ml Pefabloc SC). Centrifugation at 4°C with 21,000 rcf for 15 min separated soluble cytosolic proteins in the supernatant from the pellet. The pellet was resuspended in PBS containing 0.5% Triton X-100 and protease inhibitors and incubated for 30 min on ice. Thereby the plasma membrane as well as membranes of organelles including mitochondria, peroxisomes, endoplasmic reticulum, and golgi were solubilized. Centrifugation at 4°C with 21,000 rcf for 15 min separated soluble organelle as well as membrane proteins in the supernatant from the pellet. The pellet containing nuclear, cytoskeletal proteins, and other Triton X-100 insoluble components, was solubilized in 7.8 M urea, 2.2 M thiourea, 2% Triton X-100, and 0.1% n-dodecyl- β -D-maltoside. Any remaining insoluble contents were removed by centrifugation at 21,000 rcf for 10 min.

2.5 Cell and Islet Lysis

After removing culture media cells or islets were washed three times with PBS and lysed by adding lysis buffer (7.8 M urea, 2.2 M thiourea, and 1% Triton X-100). For two-dimensional

electrophoreses 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), and 1% IPG-ampholytes (Bio-Lyte 3/10) were added immediately before isoelectric focusing.

2.6 Two-dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed with the IEF/Criterion gel system (Bio-Rad) [39]. The first dimension used lysis buffer (above) and 11-cm nonlinear pH 3–10 immobilized pH gradient (IPG) strips. IPG strips were rehydrated with sample at 50V/14 hours, and then isoelectric focusing performed by a linear increase to 250 V over 20 min followed by a linear increase to 8000 V over 170 min and then held at 8000 V until a total of 45 kVh is reached. For the second dimension, the IPG strips were equilibrated for 12 min in 50 mM Tris/HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT, and bromophenol blue, and then 15 min in 50 mM Tris/HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2% iodoacetamide, and bromophenol blue. The strips then were embedded in 1% (wt/vol) agarose on the top of 12.5% acrylamide gels containing 4% stacking gel (Criterion gel). The second dimension SDS/PAGE was performed essentially according to Laemmli. After completion acrylamide gels were soaked 20 min in transfer buffer (25 mM Tris/HCl, 192 mM glycine, pH 8.3, and 20% methanol) and then partially electro-transferred to a Hybond-P PVDF membrane (BioRad) using a semidry transfer apparatus. The gels then were stained with colloidal Coomassie blue (GelCode blue stain).

2.7 Western Analysis

PVDF Membranes were blocked for 60 minutes by using blocking buffer (25 mM Tris, 150 mM NaCl, pH 7.5, 0.2% Tween-20, and 1.5% BSA). Membranes were then probed for 60 minutes at 25°C with a monoclonal antibody against 3-nitrotyrosine (1:5000; clone 1A6, Upstate Biotechnology), HSP60 (1:1000; 386028; Calbiochem) or cytochrome C (1:750; SC-13560; Santa Cruz Biotechnology) in blocking buffer. The membranes were then washed 4 x in washing buffer (20 mM Tris, 150 mM NaCl, pH 7.5, and 0.2% Tween-20), probed 60 min at 25°C with a goat anti-mouse antibody (horseradish peroxidase conjugate, 1:3,000, BioRad) and finally washed again 4 x in washing buffer. Immunopositive spots were visualized by chemiluminescence using ECL-Plus reagent (Amersham Biosciences) according to the manufacturer. The nitrotyrosine immunoreactivity results were verified by reduction of nitrotyrosine to aminotyrosine with sodium hydrosulfite followed by determination of remaining nitrotyrosine immunoreactivity using anti-nitrotyrosine antibody [39].

2.8 Protein Identification by MALDI-TOF Mass Spectrometry

Following 2D-SDS-PAGE proteins in gel spots matching spots immunopositive for 3-nitrotyrosine on immunoblots were subjected to in-gel tryptic digestion. The tryptic peptide mixtures were analyzed by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF/TOF PE Biosystems model 4800) as recently described in detail [39,40]. Measured peptide masses were used to search the Swiss-Prot, TrEMBL, and NCBI sequence databases using either MS-Fit (<http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm>) or Mascot (<http://www.matrixscience.com>). All searches were performed with a mass tolerance of 0.002% error (20 ppm). Only proteins that reproducibly showed positive nitrotyrosine immunoreactivity in all samples from each experimental condition were included in the analysis.

2.9 HSP60 Nitration

Recombinant human HSP60 (Stressgen) was nitrated with 5 or 25 μ M peroxyxynitrite (Calbiochem) as reported recently [41]. Briefly 0.73 mg/ml HSP60 (12.7 μ M monomer) were exposed to 5 or 25 μ M peroxyxynitrite (molar ratio 1:0.4 to 1:2, respectively) for 30 sec. at 25°

C in 100 mM potassium phosphate buffer, pH 7 with 25 mM sodium bicarbonate by rapid mixing. Then the protein solutions were stored on ice until the ATP hydrolase activity was measured. Aliquots were taken for Western analysis. The concentration of the peroxynitrite stock solution was UV spectrophotometrically determined in 0.1 M sodium hydroxide using $\epsilon = 1670 \text{ M}^{-1}\text{cm}^{-1}$ at 302 nm.

2.10 HSP60 ATP Hydrolase Activity Assay

ATP hydrolysis activity of non-nitrated and nitrated recombinant human HSP60 was measured in the presence and absence of co-chaperonin heat shock protein 10 (HSP10) and denatured mitochondrial malate dehydrogenase (mMDH) as substrate. Malate dehydrogenase (8 μM) was denatured with 6 M urea in 50 mM triethanolamine, 50 mM Tris, 20 mM MgCl_2 , 10 mM KCl, 1 mM DTT, pH 7.5 for 2 h at 25°C. For the ATP hydrolysis assay malate dehydrogenase was diluted 1:100 (final concentration 80 nM) in 20 mM MgCl_2 , 10 mM KCl, 20 mM Tris-HCl, pH 7. Then 16 nM HSP60 (final concentration oligomer) and 64 nM HSP10 (final concentration oligomer) were added and the reaction (1 h, 25°C) started by adding ATP (final concentration 1 mM). The ATPlite assay system (PerkinElmer) was used to determine the remaining ATP according to the manufacturer.

2.11 HSP60 to Proinsulin Affinity Slot-Blot Immunoassay

The affinity of native or nitrated human HSP60 towards human proinsulin (Millipore, Bedford, MA, USA) was defined by a slot immunoblotting assay. 0.75, 1.5, 3.75, and 7.5 pmol per slot of proinsulin were applied onto a PVDF membrane using the BRL Hybri-Slot manifold (Bethesda Research Laboratories, MD, USA). After releasing the vacuum and washing three times with 200 μl TBS-T (25 mM Tris, 150 mM NaCl, pH 7.5, and 0.01% Tween-20), 450 ng of HSP60 (7.76 pmol monomer, 0.55 pmol complex) were added to each slot. After a two-hour incubation with HSP60 at room temperature in the presence of 1 mM ATP in a humid chamber the membrane was washed three times with TBS-T. Then the membrane was dried and the detection of proinsulin-HSP60 complexes performed by incubation with monoclonal anti-HSP60 antibody as described earlier. HSP60 protein immobilized on the PVDF membrane was used as positive control and omission of the incubation with HSP60 and/or binding of HSP60 to immobilized BSA as negative controls.

2.12 Insulin Secretion

800,000 cells were cultivated in each well of six-well plates for four days in RPMI 1640 with 5 mM glucose and 2 mM L-glutamine. Media was changed at day two and three. Starting at day four cells were exposed to either 12 hours of alternating glucose concentrations (2 h 11 mM, 2 h 5 mM, 2 h 11 mM, 4 h 5 mM, and 2 h 11 mM) or 24 hours of a constantly elevated glucose concentration (11 mM), as used for protein tyrosine nitration experiments. The incubation with 5 mM glucose including media changes at matching time points was used as control. Following the high glucose challenges cells were washed twice with glucose free RPMI 1640 and incubated in glucose free RPMI 1640 for 30 minutes. Medium was then changed to RPMI 1640 with 2 mM L-glutamine and 11 mM glucose or 11 mM glucose in combination with 10 mM L-leucine (regular media concentration 0.38 mM) to trigger stimulated insulin release as the RIN-5F cell line does not show GSIS [42–44] like normal islet β -cells. Fractions were collected after 10 and 90 minutes, spun at 1,000 rcf for 5 min at 4°C, and kept frozen at -80°C until insulin content was measured. The remaining media was aspirated, cells washed three times with cold PBS, and lysed in 500 μl lysis buffer (10 mM KH_2PO_4 , 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P40 Substitute, 0.1% sodium deoxycholate, and 0.01% n-dodecyl- β -D-maltoside, pH 7.2) with protease inhibitors aprotinin (5 $\mu\text{g}/\text{ml}$), leupeptin (1 $\mu\text{g}/\text{ml}$), pepstatin (1 $\mu\text{g}/\text{ml}$), and Pefabloc SC (24 $\mu\text{g}/\text{ml}$). Cell lysates were assayed for protein. The amount of secreted insulin in supernatant media fractions as well as fresh media was measured

using a rat insulin 96-well plate immunoassay according to the manufacturer (ALPCO Diagnostics).

2.13 Statistics and Data Analysis

All data are presented as mean $\pm$ SEM. Unpaired two-tailed Student's *t* tests were performed using the Prism software package version 4 (GraphPad). Differences were considered significant at $p < 0.05$ and highly significant at $p < 0.02$.

3. Results

3.1 Glucose Concentration-dependent Protein Tyrosine Nitration

Our established proteomic method [39] was used to examine the effect of glucose on the nitroproteome of rat islets and the RIN-5F insulinoma cells. In β -cells as well as in islets the increase in glucose concentration from 5 mM (5.5 for islets of Langerhans) to 6.5, 8, and 11 mM resulted in a general and concentration-dependent increase in 3-nitrotyrosine immunoreactivity (Figure 1A–C islets of Langerhans, D–G islet β -cell line) and an expansion of the nitroproteome. However, the alterations caused by this regimen are highly target selective as a subset of the protein targets had a decrease or loss of immunoreactivity with increasing glucose concentrations rather than an increase (two spots indicated by arrows panel 1F and 1G).

3.2 Effect of Fluctuating Glucose Concentrations on Protein Tyrosine Nitration

To mimic physiological glucose concentration fluctuations for the RIN-5F β -cells, the cell culture was switched four to five times between media containing 5 and 11 mM glucose over a period of 12 h (Figure 2A–C). This approach revealed that fluctuations in glucose levels result in a pronounced increase in protein tyrosine nitration after repeated two-hour 11 mM glucose phases (Figure 2C). Despite the quite similar increases in nitrotyrosine immunoreactivity, the 2D-nitroproteome profiles resulting from 12 hours of intermittent exposure to 11 mM glucose versus 24 hours of continuous exposure were markedly different (compare Figure 2C and Figure 1G). Additionally, a comparison with cells grown at 5 mM glucose showed that two intermittent two-hour 11 mM glucose phases resulted in protein-specific increases in nitrotyrosine immunoreactivity that persisted even four hours after a return to 5 mM glucose in the media (Figure 2B). This particular nitroproteome pattern widely resembled the pattern observed after a third two-hour high glucose phase (compare Figure 2B and C), suggesting cumulative protein nitration. Adding the selective iNOS inhibitor L-N⁶-(1-iminoethyl)-lysine (L-NIL) [38] at the start of the 4-hour 5 mM glucose phase selectively prevented an increase in tyrosine nitration and allowed clearance or denitration [45] of nitrated proteins during the final high glucose phase (compare Figure 2B, C and D). However, L-NIL failed to prevent an increase in tyrosine nitration in a subset of proteins. This suggests a target-specific contribution of NOS toward protein tyrosine nitration in the model.

3.3 Identities of the Nitrated Proteins

Following 2D-SDS-PAGE, immunoblotting, and in gel tryptic digestion, product peptides from nitrotyrosine immunoreactive protein spots were subjected to mass spectrometric analysis. The modified proteins identified in samples from RIN-5F cells or islets that were exposed to 11 mM glucose are listed in Table 1. All proteins identified in islets were also present in the β -cell samples. The nitrated proteins participate in a variety of physiological processes, including glycolysis, mitochondrial metabolism, mitochondrial permeability, protein folding, protein translation, antioxidant defense, and signal transduction.

3.4 Effects of Cellular Stress Caused by Elevated Glucose Levels on Stimulated Insulin Secretion

Undifferentiated RIN-5F cells do not increase insulin secretion in response to glucose [44]. However, RIN-5F cells show L-leucine-stimulated insulin secretion [43,44]. Thus, we studied the unstimulated and leucine-stimulated insulin secretion (LSIS) from RIN-5F cells by collecting supernatant at 10 and 90 minutes after adding fresh media to cover any potential biphasic response known from islets [46]. This fresh media contained 11 mM glucose and 2 mM L-glutamine with and without stimulatory L-leucine. We found that 12 hours of alternating (Figure 3A and B) as well as 24 hours of permanent (Figure 3C and D) pre-exposure to elevated levels of glucose (11 mM) diminished the LSIS. This significant decrease in LSIS was observed at 10 and 90 minutes after the stimulation, but it was more pronounced at 10 minutes. We further observed a reduction in the unstimulated basal insulin release at 10 and 90 minutes. These adverse effects on insulin secretion positively correlated with the augmented protein tyrosine nitration detected earlier for the same conditions used for glucose pre-exposure.

3.5 Effects of Glucose-induced Tyrosine Nitration on Subcellular Localization of HSP60

HSP60 was nitrated under elevated glucose levels and is involved in the pathology of diabetes. Under physiological conditions HSP60 is localized in the secretory granules and mitochondria of islet beta cells but is increasingly found in the cytosol during the progression of insulinitis [47]. Therefore, we determined the subcellular localization of HSP60 in the RIN-5F cells. Differential detergent protein extraction revealed that in cells cultivated in media with 5 mM glucose the HSP60 was localized exclusively in the organelle fraction. Exposure of the cells to our physiologic glucose regimen of glucose levels alternating between 5 mM and 11 mM for 12 h had no significant influence on the subcellular localization of HSP60 (Figure 4A). The absence of cytosolic cytochrome C confirmed the integrity of the mitochondria in the organelle fraction after the first step of the differential detergent protein extraction. The increased tyrosine nitration of HSP60 observed in the 2D-nitroproteome profiles, its exclusive presence in the organelle fraction, and the dramatic increase in 3-nitrotyrosine immunoreactivity observed in the organelle fraction indicate an organelle-based HSP60 modification.

3.6 Effects of Tyrosine Nitration on HSP60 Functionality

To determine how tyrosine nitration of HSP60 affects its function, we exposed recombinant human HSP60 to the nitrating reagent peroxynitrite *in vitro*. This exposure resulted in a concentration-dependent increase in the 3-nitrotyrosine content of HSP60 (Figure 4B) that correlated with a significant decrease in the hydrolysis rate of ATP in the presence or absence of the co-chaperonin Hsp10 and/or protein substrate in the form of denatured mMDH (Table 2). The decrease in the ATP hydrolysis rate reached a maximum of -67.9 ± 1.6 % (0 vs. 25 μ M peroxynitrite) in the absence of Hsp10 and mMDH. For the complex of HSP60-Hsp10 in the presence of denatured mMDH the decrease in the ATP hydrolysis rate was still substantial but slightly less severe reaching a maximum of -55.3 ± 1.5 %. Further investigation of the interaction of native or nitrated HSP60 with HSP10 showed that increasing HSP60 nitration led to a progressive loss of the inhibitory effect of Hsp10 on the HSP60 ATP hydrolase activity. Hsp10 inhibited the ATP hydrolysis of unmodified HSP60 by -38.0 ± 1.8 % (Table 2 HSP60 vs. HSP60-HSP10), in good accordance with the literature [48]. The inhibition was reduced to -20.8 ± 2.6 % with 5 μ M peroxynitrite and diminished to $+0.4 \pm 7.8$ % with 25 μ M peroxynitrite treatment of HSP60. However, the effect of denatured mMDH on the ATP hydrolysis activity of complexes of native or modified HSP60 with Hsp10 revealed that nitrated HSP60-Hsp10 complexes partly preserve their responsiveness to protein substrates. This is illustrated by the substrate effect reaching $+60.1 \pm 4.4$ % with native versus $+41.5 \pm 5.0$ % and $+40.1 \pm 8.8$ % with nitrated HSP60, respectively (Table 2 HSP60-HSP10 vs. HSP60-HSP10-mMDH). Thus, it appeared that tyrosine nitration of HSP60 not only caused a general decrease in the ATP

hydrolysis activity but altered the interaction of HSP60 with Hsp10 and to a lesser degree with substrate proteins.

3.7 Effects of Tyrosine Nitration on Proinsulin-HSP60 Complex Formation

We next investigated the binding of HSP60 to proinsulin, which is believed to be a prerequisite step for proinsulin processing to form active insulin [49,50]. Using immunodetection and optical densitometry we determined that 7.5 pmol of immobilized proinsulin bound virtually all the added native HSP60 (nHSP60, 530 fmol complex), whereas substantially less nitrated HSP60 (nY-HSP60) was bound (Figure 4C). We observed a positive correlation between the amount of immobilized proinsulin and the bound native HSP60. In contrast, below 7.5 pmol of immobilized proinsulin the amount of bound nitrated HSP60 was very small and became independent of the amount of proinsulin. Without exposure to HSP60, there was no immunoreactivity to immobilized proinsulin expressed ruling out nonspecific immunoreactivity. Positive controls with immobilized native and nitrated HSP60 showed that they have the same HSP60 immunoreactivity, which ruled out alteration of antibody-antigen interaction by HSP60 nitration. The very minor HSP60 immunoreactivity resulting from the exposure of immobilized BSA to HSP60 further ruled out any relevant contribution of the BSA in the proinsulin stock solutions. Together, our data clearly demonstrate a significant decrease in the binding efficiency of nitrated HSP60 towards proinsulin.

4. Discussion

Inadequate control of blood glucose is the key factor of IFG, IGT, and diabetes. It is characterized by hyperglycemia especially after meals and elevated blood proinsulin levels reflecting the progression of β -cell dysfunction and insulin resistance [36,51,52]. Acute or chronically elevated glucose can cause toxicity that correlates with oxidative stress, a mediator in the pathogenesis of diabetes-associated complications [12,18]. Thereby understanding the different effects of oxidative stress on beta cell function and insulin secretion are essential [15,24,53]. These conditions favor oxidative protein modifications like protein nitration, the extent of which potentially covers the entire range from a transient adaptive response based on regulated nitration/denitration to excessive and potentially accumulative modification due to overwhelmed cellular response mechanisms [45]. In this context our study represents the first systematic investigation that correlates glucose concentrations, protein tyrosine nitration, and insulin secretion in pancreatic islets and β -cells. Our data showed that the extent of cellular tyrosine protein nitration, along with an expansion of the nitroproteome, mirrored rises in the glucose concentration during continuous exposure as well as during the simulation of a more physiologically relevant periodic fluctuation in the glucose concentration. In the following paragraphs we discuss the possible impact of these alterations.

Mitochondria and secretory vesicles

Our results indicate that mitochondria are central targets of glucose-induced protein nitration. Identified nitrated mitochondrial proteins include those involved in protein folding, metabolic functionality, antioxidative capacity, and membrane permeability (Figure 5, Table 1). While the impairment of protein folding and antioxidative capacity will be detrimental, the alterations in metabolic functionality and membrane permeability could be part of normal adaptive mechanisms at least at low levels of modification. All together they potentially contribute to an altered stimulated insulin response in β -cells. As almost all proteins of the mitochondrial proteome have to be imported, accurate refolding after import is crucial for mitochondrial function and biogenesis. In the mitochondrial matrix this is accomplished through the two major mitochondrial chaperone classes, heat shock proteins 70 (HSP70) and HSP60s [54]. Their ATP-dependent folding most likely occurs in a cooperative and sequential manner. Additionally, the HSP60-HSP10 chaperone system may prevent protein misfolding under

stress conditions and facilitate the unfolding, refolding and proper assembly of nonnative proteins, thus preventing their aggregation [55]. The physiological importance of HSP60-dependent protein folding is illustrated by a defect found in HSP60 leading to spastic paraplegia-13 [56].

However, most mechanistic studies have been performed with the highly comparable bacterial homologs GroEL (HSP60) and GroES (HSP10). To provide proper function the two rings of GroEL oscillate between binding-active and folding-active modes out of phase with each other [57–60]. This process is characterized by sequential allosteric transitions and depends on the binding and hydrolysis of ATP as well as the interaction with GroES and substrate. Thus, the significant nitrative inhibition of the HSP60 ATPase activity observed in our study implicates a fundamental loss of function that could include one or more of the following: (i) ATP binding; (ii) ATP hydrolysis; and (iii) interactions between amino acids involved in crucial conformational shifts, binding of HSP10, and/or binding of substrate.

The sites for co-chaperonin and substrate binding are both localized in the apical domains of GroEL and HSP60 [61]. The sites partly overlap and consist of mostly hydrophobic amino acids including the highly conserved Tyr222 (Tyr199 in GroEL) and Tyr226 (Tyr203 in GroEL). An involvement of the apical domain was substantiated by (i) the impaired interaction between HSP60 and HSP10 manifested in the nitrative loss of HSP60 ATPase inhibition by HSP10 in the absence of protein substrate; and (ii) the decreased stimulatory effect of denatured mMDH on the ATPase activity of the HSP60-HSP10 complex. The interaction with denatured mMDH affected nitrated HSP60 even in the absence of HSP10 as it promoted the inhibition of nitrated HSP60. Since the correct initial substrate interaction depends on a continuous hydrophobic interface formed by up to four cis apical domains, any change in the symmetry and hydrophobicity of the binding interface can abolish substrate binding [62]. Nitration of one or both of the tyrosine residues of the substrate binding site in the apical domains [61] could be responsible for such a change, as nitration decreases the pKa of the phenolic group and therefore the hydrophobicity of tyrosine [63]. Consistent with this prediction, a recent study showed that treatment of GroEL with peroxynitrite results in nitration of Tyr203 and inactivation of its refolding activity [64]. Khor et. al. also found that while hydrogen peroxide did not affect activity of the GroEL, high concentrations of HOCl and peroxynitrite inhibited the enzyme. With HOCl extensive oxidatization of methionines and cysteines occurred and this was less widespread with peroxynitrite. Additionally, the extent of methionine modification is lower in this current study since we used 20 fold less peroxynitrite and incubations were done in the presence of bicarbonate buffer, that cause a shift from oxidative reactions to tyrosine nitration. HOCl caused GroEL conversion from oligomers to the inactive monomers and this was not observed with peroxynitrite. This suggests that the mechanism of inactivation by HOCl, that causes mainly methionine/cysteine oxidation, is different from that of peroxynitrite especially at low concentrations. However, it is still possible that oxidations of these amino acids affect the overall activity of HSP60 [64]. An altered mitochondrial protein folding due to HSP60 dysfunction will have pathological consequence on the proteome integrity. This potentially includes increased turnover or even aggregation of denatured mitochondrial proteins especially under stress conditions consequently resulting in reduced mitochondrial functionality in β -cells.

The effects of HSP60 nitration in β -cells could reach beyond mitochondria, as HSP60 has also been shown to be present in proinsulin-containing immature secretory granules where HSP60 associates with proinsulin and convertase PC1 [49,50]. This contributes to the conversion of proinsulin into insulin. The substantial loss of interaction between HSP60 and proinsulin upon nitration of HSP60 observed by us *in vitro* could directly affect the processing of proinsulin to insulin in secretory granules and therefore insulin secretion. The comparable decrease in both,

basal and L-leucine-stimulated insulin secretion in RIN-5F cells [43,44] following glycemic stress demonstrated here supports this conclusion.

Metabolic changes in beta cell function

Mitochondria play an important role in regulation of insulin. Glucose metabolic intermediates, the structural integrity and proton gradient are major determinants of insulin secretion [65]. In β -cells any significant change in mitochondrial oxidative metabolism would also affect cytosolic glycolysis and vice versa. This very tight coupling is essential for the maintenance of the high rate of glucose metabolism that finally translates into the metabolic coupling of the insulin release [66]. Thus, effects of the glucose-dependent oxidative modifications of mitochondrial metabolic enzymes on the generation of ATP and secretagogues may well translate into an altered insulin secretion and ultimately contribute to β -cell apoptosis [66, 67]. The glycolytic pathway is prime target of glucose-induced tyrosine nitration in pancreatic islets and β -cells. We have recently shown that nitration of aldolase A inhibits enzyme activity [41] along with simultaneous nitration of other downstream enzymes, in particular G3PDH and phosphoglycerate kinase, can lead to decrease glycolytic flux and so affect intermediates available to the mitochondrial tricarboxylic acid (TCA) pathway. The TCA cycle enzymes malate dehydrogenase and aconitase are among the identified tyrosine nitrated proteins. Both of these enzymes have been demonstrated to be affected by oxidative modifications which inhibit malate dehydrogenase [68] and contributes to the irreversible oxidative inactivation of aconitase [69,70] by the loss of labile iron from the $[4\text{Fe-4S}]^{2+}$ cluster. It is clear that in an environment in which enhanced protein nitration occurs, increased modification of oxidation-sensitive chemical groups like thiols would also take place. This damage may exacerbate the antioxidant potential of an already low intrinsic antioxidant capacity of β -cells [26,27]. Additionally, factors such as inactivation by tyrosine nitration of MnSOD and, reduced levels of mitochondrial NADPH and glutamate that are crucial for antioxidant systems like glutathione/glutathione peroxidase/glutathione reductase [71,72] may enhance oxidative stress. A decrease in the activity of the TCA cycle enzymes may slow down the oxidation of glucose carbons in the mitochondria and therefore decrease the generation of GTP as well as NADH and FADH_2 [66,67]. This will affect the activity of the electron transport chain and therefore membrane hyperpolarization and ATP synthesis. Nitration triggered changes in the activity and/or regulation of glutamate dehydrogenase (GDH), glutamate oxaloacetate transaminase 2 (GOT2) [73], and L-hydroxyacyl-CoA dehydrogenase may contribute to these alterations by affecting the amino acid metabolism, glutamate - 2-oxoglutarate (α -ketoglutarate) balance, and fatty acid β -oxidation (Figure 5). However, the energetic effects might be accompanied by a shift in the relative amounts of metabolic intermediates. A substantial shift in these intermediates would influence the regulation of the TCA cycle and pyruvate metabolism as well as alter the coupling of the glycolytic NADH production with the mitochondrial respiration through the malate-aspartate shuttle and the cytosolic NADPH regeneration through the pyruvate-malate shuttle. The glucose-mediated nitration of metabolic proteins could be part of the regulation as well as the impairment of the stimulated insulin secretion of β -cells [66,74,75]. This would include stimulating substances like amino acids due to our observation as a pre-exposure to glycemic stress diminishes LSIS. Consistent with this observation, the stimulatory effect of L-leucine has been attributed to its mitochondrial metabolic breakdown and allosteric regulation of GDH in the presence of L-glutamine [42], which are potentially altered by nitration of mitochondrial proteins identified by us (Figure 5).

The permeability of the inner and outer membrane is indispensable for the morphological and functional integrity of mitochondria and may be affected by oxidative damage. Voltage-dependent anion-selective channels (VDAC), a main interface between mitochondrial and cellular metabolism in the outer membrane, form complexes with the adenine nucleotide translocator (ANT) and various peripheral kinases including hexokinase/glucokinase and

creatine kinase [76]. This leads to a tight functional coupling that plays a crucial role in regulation of mitochondrial permeability and therefore Ca^{2+} homeostasis and metabolic channeling of metabolites and ATP [65,77]. The alteration of the permeability of VDACs for ADP and ATP at and beyond contact sites between the outer and inner membrane may stimulate oxidative phosphorylation and promote insulin release in glucose-stimulated β -cells [65,78]. The effect of tyrosine nitration on VDACs in β -cells is yet unknown but it could alter the regulatory properties of tyrosine phosphorylation [79] in the case of VDAC1 [80] and VDAC2 [81]. Tyrosine nitration could also alter the interactions of VDAC with proteins like glucokinase, ANT, and/or the proapoptotic molecules BAK and BAD [65,82]. This may contribute to changes in the metabolic and regulatory function of mitochondria including the membrane potential homogeneity [83].

5. Conclusion

The cellular processes involved in the fuel sensing and stimulated insulin secretion of islet β -cells are very complex and only partly understood. In this context our study delineates for the first time the potential (patho)physiological impact of glucose-triggered alterations in the nitroproteome of islets of Langerhans and β -cells. Thereby five factors are of special importance: (i) the presence of nitrated proteins even under normal glucose concentrations; (ii) the physiological functions of the identified target proteins, which include the metabolic activity, protein folding, membrane permeability, signal transduction, and antioxidant capacity (Figure 5); (iii) the influence of protein nitration on protein function; (iv) the impaired basal as well as stimulated insulin secretion in correlation with augmented protein nitration shown in this study and others [33]; and (v) recent evidence supporting the hypothesis that protein tyrosine nitration is a regulated process [45]. The combination of these factors implicates that tyrosine nitration may indeed represent a significant novel regulatory mechanism for the metabolic coupling of β -cell insulin secretion and thus supports (patho)physiological effects of protein nitration on the function of β -cells. The possible impairment of the dynamic regulation of the nitroproteome by augmented oxidative stress due to glucose toxicity inflicted by frequent episodes of severe hyperglycemia would provide a mechanism for the detrimental effects of hyperglycemia on β -cells. This could therefore help to explain the molecular mechanism for the impaired glucose disposal in individuals with IGT alone or a combination of IFG and IGT [36]. An impaired cellular regulation of the nitroproteome could also explain the accumulation of nitrated proteins in β -cells during the onset and progression of diabetes [28–30] and represent a potential target for therapeutic interventions in β -cell dysfunction.

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Abbreviations

IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IL-1β	interleukin-1 β
iNOS	inducible nitric oxide synthase

RNS	reactive nitrogen species
NO	nitric oxide
SOD	superoxide dismutase
GSIS	glucose-stimulated insulin secretion
LSIS	leucine-stimulated insulin secretion
HSP60	heat shock protein 60
HSP10	heat shock protein 10
L-NIL	L-N6-(1-iminoethyl)-lysine
GDH	glutamate dehydrogenase
GOT2	glutamate oxaloacetate transaminase 2
ANT	adenine nucleotide translocator
mMDH	mitochondrial malate dehydrogenase
VDAC	voltage-dependent anion-selective channel
nHSP60	native HSP60
nY-HSP60	nitratated HSP60

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Figure 1. Glucose concentration dependent increase in protein tyrosine nitration

Representative 2D-blot for 3-nitrotyrosine immunoreactivity of islet β -cells and islets of Langerhans sampled after 24 h of exposure to the specified glucose concentrations. Cells have been cultured at 5 mM glucose prior to the experiment. (A–C) islets of Langerhans exposed to 5.5 (A), 8 (B), and 11 (C) mM glucose. (D–G) islet beta cells exposed to 5 (D), 6.5 (E), 8 (F), and 11 (G) mM glucose. In most cases nitrotyrosine immunoreactivity increased with increase glucose concentrations, however in a few proteins (see arrows panel F and G) immunoreactivity decreased.

Figure 2. Increase in protein tyrosine nitration by fluctuating glucose levels

Representative 2D-blot for 3-nitrotyrosine immunoreactivity of islet β-cells (A–D) sampled after the exposure to the specified glucose concentrations. Cells have been cultured at 5 mM glucose prior to the experiment. Conditions for glucose exposure were: (A) 12 h 5 mM with media change after 2, 4, 6, and 10 hours; (B) 2 h 11 mM, 2 h 5 mM, 2 h 11 mM, and 4 h 5 mM; (C) 2 h 11 mM, 2 h 5 mM, 2 h 11 mM, 4 h 5 mM, and 2 h 11 mM; (D) 2 h 11 mM, 2 h 5 mM, 2 h 11 mM, and 4 h 5 mM glucose + 50 μM L-NIL; (E) schematic time dependent glucose profiles of (B) and (C). The selective effect of L-NIL is illustrated by o for prevention of an increase in nitration, + for missed prevention of an increase in nitration, and – for allowing clearance/denitration of nitrated proteins.

Figure 3. Function of nitrated proteins and stimulated insulin secretion

Influence of the pre-exposure to 12 hours of alternating glucose levels - 2 h 11 mM, 2 h 5 mM, 2 h 11 mM, 4 h 5 mM, and 2 h 11 mM - (B1, B2) or 24 hours of constantly elevated - 11 mM - glucose levels (B3, B4) on basal and L-leucine stimulated insulin secretion. Pre-exposure with media containing 5 mM glucose was used as controls. Insulin secretion was measured at 10 (B1, B3) and 90 minutes (B2, B4) after adding fresh media with or without 10 mM leucine. Relative amounts of secreted insulin of three independent experiments (n=3) are shown. Fresh RPMI 1640 media did not contain any relevant amounts of insulin (data not shown). Asterix used are * for the comparison between insulin secretion after high glucose stress and controls and # for the comparison between basal and stimulated (10 mM L-leucine) insulin secretion. Thereby double asterix represent alterations with $p < 0.02$ and single asterix $p < 0.05$.

Figure 4. Protein tyrosine nitration of HSP60

(A) Subcellular localization: Representative 1D-blot images for 3-nitrotyrosine, HSP60, and cytochrome C of islet beta cells after differential detergent protein extraction showing immunoreactivity in cytosol (cyt), organelle/membrane (o/m), and nuclear/cytoskeletal (n/c) fraction. Protein content of fractions was measured and equal amounts of protein were loaded for each fraction. Cytochrome C distribution was used to control mitochondrial integrity in the organelle/membrane during fractionation. (B) *In vitro* nitration: The graph shows the quantification of 3-nitrotyrosine immunoreactivity of artificially nitrated HSP60 using peroxynitrite. Thereby asterix represent alterations with $p < 0.05$. (C) Effect of protein tyrosine nitration on HSP60 proinsulin interaction: Representative slot blot illustrating the

differences between the binding of nHSP60 and nY-HSP60 HSP60 to immobilized proinsulin (PI) at various molar ratios.

Figure 5. Scheme of physiological function of identified nitrated proteins in β-cells

These proteins are: 1 aldolase, 2 glyceraldehyde-3-phosphate dehydrogenase, 3 phosphoglycerate kinase, 4 malate dehydrogenase, 5 glutamate oxaloacetate transaminase, 6 aconitase, 7 glutamate dehydrogenase, 8 VDAC, 9 manganese superoxide dismutase, 10 3-hydroxyacyl-CoA dehydrogenase; abbreviations: Ac-CoA acetyl-CoA, Asp aspartate, 1,3-BPG 1,3-bisphosphoglycerate, DHAP dihydroxyacetone phosphate, F-1,6-P fructose-1,6-bisphosphate, F-6-P fructose-6-phosphate, G-6-P glucose-6-phosphate, Glu glutamate, Gln glutamine, G-3-P glyceraldehyde-3-phosphate, α-KG α-ketoglutarate, KIC α-ketoisocaproic acid, OOA oxaloacetate, PEG phosphoenolpyruvate, 2-PG 2-phosphoglycerate, 3-PG 3-phosphoglycerate, Pyr pyruvate, Suc-CoA succinyl-CoA;

Table 1

Identification of nitrated proteins

The rat islet of Langerhans and islet beta cell proteins immunopositive for 3-nitrotyrosine were identified by mass spectrometry. Values for sequence coverage and peptide matches originate from Mascot search using MALDI-TOF mass spectrometry peptide map data. Only proteins reproducibly present in the 2D-nitroproteome profiles of all samples from each experimental condition using 11 mM glucose are listed for islet β -cells and islets of Langerhans.

Source	Protein	% Sequence coverage	Peptide matches
RIN-5F cells	Aconitase 2 (mitochondrial)	36	23
	Activated protein kinase C receptor (RACK 1)	35	8
	Aldolase A	26	6
	Eukaryotic translation elongation factor 1 α 1	29	12
	Glutamate dehydrogenase	26	7
	Glutamate oxaloacetate transaminase 2 (mitochondrial)	48	10
	Glyceraldehyde-3-phosphate dehydrogenase (G3PDH)	32	6
	Heat shock protein 60 (HSP60)	29	9
	Heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP-A2/B1)	44	12
	L-3-Hydroxyacyl-CoA dehydrogenase	62	14
	Manganese superoxide dismutase (MnSOD)	19	3
	Malate dehydrogenase	35	8
	Phosphoglycerate kinase	30	9
	Protein disulfide isomerase (ER60)	27	12
	Voltage-dependent anion-selective channel protein 1, 2, and 3 (VDAC-1,-2,-3)	30, 28, 34	7, 5, 6
Rat islets	Aldolase A	21	5
	Eukaryotic translation elongation factor 1 α 1	12	4
	Glutamate oxaloacetate transaminase 2 (mitochondrial)	13	5
	Glyceraldehyde-3-phosphate dehydrogenase (G3PDH)	18	4
	L-3-Hydroxyacyl-CoA dehydrogenase	29	6
	Malate dehydrogenase	21	5

Table 2
Effect of protein tyrosine nitration on HSP60 ATPase activity

The ATP hydrolase activity of HSP60 is shown in dependence of the applied peroxynitrite concentration and the interaction with its co-chaperonin HSP10 and/or protein substrate denatured mitochondrial malate dehydrogenase (mMDH). The data are represented as mean ± SEM of 4 independent experiments (n = 4).

Peroxyntirite μM	HSP60	HSP60-HSP10	HSP60-mMDH	HSP60-HSP10-mMDH
0	20.64 ± 1.32	12.77 ± 0.74 [#]	21.99 ± 1.32	20.36 ± 0.80 [•]
5	10.74 ± 0.52 [*]	8.48 ± 0.30 ^{*,#}	7.36 ± 0.29 ^{*,#}	12.00 ± 0.61 ^{*,•}
25	6.57 ± 0.11 [*]	6.62 ± 0.61 [*]	3.94 ± 0.34 ^{*,#}	9.11 ± 0.34 ^{*,#,•}

Values are shown as μM ATP/min/μM HSP60. Asterixis used are

^{*} for the comparison between ATP hydrolase activity untreated (0 μM peroxynitrite) and treated HSP60 for a certain condition,

[#] for the comparison between ATP hydrolase activity of untreated or treated HSP60 alone and in combination with mMDH or HSP10, and

[•] for the comparison between ATP hydrolase activity of untreated or treated HSP60 in combination with HSP10 in the presence and absence of mMDH, respectively. Thereby asterixis represent alterations with p < 0.02 and were therefore considered significant.