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Mechanisms Underlying Resistance of Pancreatic Islets from ALR/Lt Mice to Cytokine-Induced Destruction

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Nuclear and mitochondrial genomes combine in ALR/Lt mice to produce systemically elevated defenses against free radical damage, rendering these mice resistant to immune-mediated pancreatic islet destruction. We analyzed the mechanism whereby isolated islets from ALR mice resisted proinflammatory stress mediated by combined cytokines (IL-1β, TNF-α, and IFN-γ) in vitro. Such damage entails both superoxide and NO radical generation, as well as peroxynitrite, resulting from their combination. In contrast to islets from other mouse strains, ALR islets expressed constitutively higher glutathione reductase, glutathione peroxidase, and higher ratios of reduced to oxidized glutathione. Following incubation with combined cytokines, islets from control strains produced significantly higher levels of reactive nitrogen and NO than islets from ALR mice. Nitrotyrosine was generated in NOD and C3H/HeJ islets but not by ALR islets. Western blot analysis showed that combined cytokines up-regulated the NF-κB inducible NO synthase in NOD-Rag and C3H/HeJ islets but not in ALR islets. This inability of cytokine-treated ALR islets to up-regulate inducible NO synthase and produce NO correlated both with reduced kinetics of IκB degradation and with markedly suppressed NF-κB p65 nuclear translocation. Hence, ALR/Lt islets resist cytokine-induced diabetogenic stress through enhanced dissipation and/or suppressed formation of reactive oxygen and nitrogen species, impaired IκB degradation, and blunted NF-κB activation. Nitrotyrosylation of β cell proteins may generate neoantigens; therefore, resistance of ALR islets to nitrotyrosine formation may, in part, explain why ALR mice are resistant to type 1 diabetes when reconstituted with a NOD immune system. The Journal of Immunology, 2005, 175: 1248–1256.

Among the various endocrine cell types present in murine pancreatic islets, the β cells are particularly susceptible to free radical-mediated stress because of low glutathione content and low activities of the antioxidant enzymes that produce it (1, 2). The cytopathic contributions of free radicals to the development of autoimmune type 1 diabetes (T1D) in the NOD mouse have been clearly illustrated by comparison to the closely related but T1D-resistant ALR/Lt strain. ALR/Lt mice and NOD/Lt mice have a common ancestry and a high degree of gene sharing, including an extensive region of the MHC (3–5). ALR mice were originally selected for cell resistance to chemical diabetes produced by alloxan, a potent generator of hydroxyl radicals (6). However, subsequent studies showed that resistance was systemic, entailing increased activities of antioxidant enzyme activities in multiple tissues and higher tissue ratios of reduced to oxidized glutathione (7). ALR islets in vivo were resistant to T cell-mediated autoimmune attack following lethal irradiation and chimerization with NOD bone marrow or following adoptive transfer of NOD CTL clonotypes (8). In vitro, ALR islets were distinguished from NOD islets both by resistance to lysis by islet-reactive NOD CTL clonotypes and also by maintaining viability and insulin secretory function following combined cytokine treatment (8). Genetic analysis has shown that the T1D resistance of ALR mice and their β cells in vivo entails both nuclear and mitochondrial genomic contributions (4, 5).

In the NOD mouse, cytokines produced by pancreatic islet infiltrating immune cells are important mediators of islet β cell damage when present either alone or in combination (9, 10). Incubation of mouse pancreatic islets in vitro with the combination of IL-1β, TNF-α, and IFN-γ leads to the formation of toxic nitrogen and oxygen radicals, most of which are generated by the β cells themselves (11). Islet viability after cytokine exposure can be significantly increased by scavenging oxidative radicals (12, 13) and by inhibiting the generation of nitrogen-based radicals (14). Peroxynitrite (ONOO−), a highly reactive radical species produced by the reaction of NO with superoxide (15–17), is a more potent oxidant with increased cytotoxic potential compared with either NO or superoxide alone (18–20). The importance of both nitrogen and oxygen free radicals in cytokine-mediated β cell destruction is evidenced by studies of islets isolated from mice with a null mutation in the gene encoding inducible NO synthase (iNOS). These islets show short-term (48 h) but not long-term (9 days) resistance to proinflammatory cytokine-mediated toxicity (21, 22). We have also recently demonstrated that when human islets are treated in vitro with cytokines as well as with the iNOS inhibitor, 1-N4-monomethylarginine, there was a measurable decrease in
Poninflammatory cytokines activate NF-κB via MyD88/cytokine receptor interactions (24). The role of inhibitor of κB kinase β (IKKβ) is critical for NF-κB activation via the phosphorylation and degradation of the NF-κB inhibitory subunit IκBκ. Phosphorylation of IκBκ affects its dissociation from a complex with NF-κB p50 and p65 subunits, allowing them to dimerize and translocate into the nucleus to initiate transcription of iNOS and other inflammation-associated responses (25–27). Although the NF-κB-dependent pathway for generating NO by iNOS induction is established, the mechanism for superoxide/hydrogen peroxide generation by islets exposed to cytokines is unknown. Cytokines have been shown to induce mitochondrial dysfunction (28), potentially leading to an increased superoxide leak from the electron transport chain and resulting in oxygen radical accumulation inside of islet cells (23). ONOO− generated within the β cell when superoxide and NO radicals combine lead to protein nitrosylation, reduced insulin stores, and β cell death. Therefore, islet β cell survival would require blocking the production of both NO and superoxide/hydrogen peroxide.

In this report, we provide a detailed investigation into the mechanism underlying the up-regulated islet β cell stress response system of ALR mice that confers such strong resistance to cytokine-mediated toxicity. Our results not only confirm the contributions of increased glutathione recycling enzyme activities in maintaining β cell viability but also show that when cytokine stimulation, ALR islets fail to activate NF-κB and stimulate iNOS production. Hence, detoxification of cytokine-induced superoxide, coupled with the inhibition of iNOS and NO production, prevents ONOO− formation and nitrosylation reactions, thereby protecting pancreatic islet β cells in the TID-resistant ALR/Lt mouse.

Materials and Methods

Mice

For studies entailing endogenous free radical generation in cytokine-treated islets, NOD/MrkTac and C57BL/6 mice were purchased from Taconic Farms, and ALR/Lt female mice were obtained from The Jackson Laboratory. For other studies, NOD/Lt female mice and immunodeficient NOD.129S7(B6)−Rag1−/−LysM−/− mice were purchased from The Jackson Laboratory, as were ALR/Lt female mice. Mice were housed and fed under specific pathogen-free conditions and were cared for according to the guidelines of either the Canadian Council on Animal Care or the Institute of Laboratory Animal Resources.

Islets and islet cells

Pancreatic islets were isolated from ALR, NOD, and C57BL/6 mice, ages 4–6 wk, by collagenase digestion of the pancreas and Ficol density gradient purification followed by hand picking of the isolated islets (29). For some experiments, the islets were dispersed into single cells by incubation for 10 min at 37°C in Ca++− and Mg++−free PBS containing 0.2 mg/ml EDTA followed by syringe injection through progressively narrower gauge needles from sizes 16 to 22 μm.

Islet and islet cell incubations

Islets (500–1000) were incubated in 1.6 ml of medium in 35 × 10-mm Falcon tissue culture dishes (BD Biosciences). Islet cells (105) were incubated in 170 μl of medium in 96-well tissue culture plates (A/2; Sarstedt). For immunohistochemical studies, islet cells (105) were seeded in 10 μl of medium in 5-mm well tissue culture chamber slides (Lab-Tek II; Nalge Nunc International) and incubated for 30 min at 37°C in 5% CO2 to allow the cells to attach to the slides before adding 200 μl of medium. Islets and islet cells were preincubated for 48 h at 37°C in 5% CO2 in RPMI 1640 medium (Invitrogen Life Technologies) containing 11 mM d-glucose and supplemented with 2% BSA, 100 μM penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 12 mM HEPES (test medium). Islets and islet cells were incubated in test medium without (vehicle) and with the cytokine combination of IL-1β (30 U/ml), TNF-α (10 U/ml), and IFN-γ (10 U/ml) for 72 h at 37°C in 5% CO2. Human rIL-1β (2–4 × 10−7 U/ml) was kindly provided by Upjohn (Kalamazoo, MI); murine rTNF-α (1.2 × 10−7 U/ml) and murine rIFN-γ (8 × 10−8 U/ml) were provided by Genentech. After 72 h, incubation media were collected for some assays, and islets and islet cells were washed three times in PBS and submitted to other assays.

MTT assay

Islet cell viability was determined by a colorimetric assay that detects the reduction of MTT (Sigma-Aldrich) into a blue formazan product (30, 31).

Insulin assay

Insulin was extracted from islets by incubation in acidified ethanol (75% ethanol, 1.5% 12 mM HCl, and 23.5% H2O) for 18 h at 4°C. The ethanol extracts of islets were diluted in insulin assay buffer, and insulin was measured byRIA using rat insulin standard (Linco Research).

Hydrogen peroxide assay

Hydrogen peroxide in islet incubation medium was measured by spectrophotometry using an assay based on the peroxidase-oxidase reaction and able to measure hydrogen peroxide concentrations as low as the nanomolar range (32).

Glutathione assays

Glutathione and glutathione disulfide (GSSG) contents in islets were measured by a HPLC method and postcolumn derivatization with ortho-phthaldialdehyde followed by fluorescence detection (33).

Nitrite assay

Nitrite, the stable end product of NO in aqueous solution, was measured in islet incubation medium by an on-line semiautomated procedure using HPLC (34), modified as reported previously (12).

Nitrotyrosine (NT) assay

Sample preparation was described previously (35). Islets were briefly sonicated in 400 μl of sodium acetate (10 mM, pH 6.5) and then rapidly vortexed for 1 h and centrifuged for 10 min at 12,000 × g. A 50-μl aliquot of the supernatant was removed for protein assay by bicinchoninic acid method (Pierce). One hundred fifty microliters of the supernatant was added to 25 μl of sodium acetate buffer and 50 μl of pronase (1 mg/ml in acetate buffer). The solution was then heated at 50°C for 18 h and dried in an Speed Vac system. The dried extract was dissolved in 100 μl of ethanol. H2O2 (70:30) by rapid vortexing and then centrifuged at 12,000 × g for 10 min. The supernatant was frozen at −20°C until derivatization and quantitation by HPLC, as described previously (36). Derivatization of NT was performed by adding 10 μl of sodium borate (0.1 M [pH 8.7]) and 1 μl of 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (10 mg/ml in ethanol) to 50 μl of ethanol:H2O solution containing islet extract and incubating at 60°C for 2 min. The reaction was terminated by the addition of 15 μl of 0.1 M HCl and an aliquot (50–80 μl) was injected into the HPLC column. The chromatography method was as described previously (36). The detection limit for NT was −1 pmol at a signal-to-noise ratio of 5.

Antioxidant enzyme activity

Antioxidant enzyme activities were assayed in freshly isolated islets from ALR and NOD.Rag1 male mice. The peroxide dissipating enzyme glutathione peroxidase (GXP) was assayed at 25°C using the Bioxytech Gpx-340 assay kit (OxisResearch), and the activity of glutathione reductase (GSR) was assayed at 25°C with the Bioxytech GR-340 assay kit (OxisResearch). Both assays were run as per the manufacturer’s directions. Enzyme analyses were performed in Costar flat-bottom 96-well microtiter plates in a total volume of 200 μl. Changes in absorbance were read in a SpectraMax Microplate Spectrophotometer with SOFTmax PRO for Macintosh ( Molecular Devices). All readings were normalized by protein content (milligrams), which was determined using Total Protein Reagent (Sigma-Aldrich).

Gene expression

RNA was purified from isolated islets using TRizol Reagent (Invitrogen Life Technologies). cDNA was generated using the Superscript Choice
System (Invitrogen Life Technologies). Primer sets, specific for the genes of interest, were validated before use by sequencing of the amplified PCR product to determine specificity. The following primers were used: Gpx1 (Gpx1.1, CCGCTACcATcAGITGgAC, and Gpx1.2, TAAGAgCCgTTgAgCCTT); Gsr (Gsr.F, TgAcCTCTAgTgAAgCTC, and Gsr.R, gAATgTgCTgAATgCCgTGa); INS2 (Ins2.F, AgCAAcAggAgCT CTAITC, and Ins2.R, TgCCAggCTgTCTAggCTCA); and Ikbα (Nkb.B, CCGACACAggCTAgCCgCCTAggCTC, and Nkb.B.2, TgAgCgCgggCCgCgCCTAggCTC). Amplification using a primer set specific for 18S rRNA (18S.F, CgAgCAgCTAggAAAAgAgATAggAT, and 18S.R, CgAcAACCCTcCgAgTTCgT) was used for normalization. The tests were run using three pools of islet cDNA per group. These tests were run on an ABI Prism 7700, using SYBR Green JumpStart Taq Ready Mix (Sagra-Detection) for per the manufacturer’s protocol. Final reaction contents were 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.5 mM MgCl2, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP, 0.2 mM dCTP, 0.2 mM dTTP, 0.2 mM dCTP, 0.2 mM dTTP, 13.2 mM of each primer, 0.625 U of TaqDNA Polymerase, Jump Start Taq Ab, SYBR Green I, and 5 µl of template cDNA in a total volume of 50 µl. All PCR conditions were 2 min of an initial denaturation step at 94°C, followed by 45 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s, and then finally a 4°C hold.

For determination of IKKβ (Ikkβ) expression in islets and liver we used: IKKβ (Ikkβ.B, TCTCTgAggAATgCTgCgTgCTgC, and Ikkβ.R, TgTg gTgACAggCcAggCC), and 18S rRNA as a control. Islet donors were either ALR/LJ or NOD/Rag. PCR was run in an ABI Gene Amp PCR System 9700. Final reaction contents were 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP, 0.2 mM dCTP, 13.2 mM of each primer, 0.5 U of TaqDNA Polymerase, Jump Start Taq Ab, SYBR Green I, and 5 µl of template cDNA in a total volume of 25 µl. PCR conditions were as described above.

**Immunohistochemical studies**

Islet cells attached to tissue culture chamber glass slides were fixed with 4% paraformaldehyde in PBS for 10 min and washed twice in PBS. The slides were stored at −70°C until cell staining was performed, as described previously (37). Briefly, the fixed cells were permeabilized with 1.5% saponin in PBS (PBS-saponin). Endogenous cell permeabilization was blocked by incubation in PBS-saponin containing 1% H2O2, followed by 20% normal goat serum diluted 1/20 in PBS. The cells were incubated first with 10 µg/ml rabbit anti-anti-TNF-α Ab (Upstate Biotechnology). Control incubations were performed with rabbit isotype-matched IgG (Cedarlane Laboratories) and a mixture of anti-NT Ab and 10 mM 3-nitro-1-tyrosine (Aldrich) to neutralize the anti-NT Ab. Next, the cells were incubated with a secondary Ab, biotinylated goat anti-rabbit IgG (Zymed Laboratories), and then with streptavidin-peroxidase conjugate (Zymed Laboratories) and substrate chromogen, 3-amino 9-ethylcoupling (Biocare). Additional incubation with 10 µg/ml rabbit anti-NT Ab (Cell Signaling Technology), anti-iNOS Ab, anti-CREB, and anti-IκBα Ab (Cell Signaling Technology), followed by HRP-conjugated Ig and detection with ECL (Amersham Biosciences). Western blot analysis was performed with anti-iNOS Ab (Cayman Chemical), anti-IκBα Ab (Santa Cruz Biotechnology), and anti-IκBβ (Cell Signaling Technology), followed by HRP-conjugated Ig and detection with ECL (Amersham Biosciences). Western blot analysis for both iNOS, IκBα, and IκBβ were performed in triplicate for each time point.

**Statistical analysis**

Data are expressed as means ± SE, and the statistical significance of differences between mean values was determined by Student’s unpaired t test. Values for p < 0.05 were considered significant.

**Results**

**Proinflammatory cytokine-induced pancreatic islet dysfunction**

In vitro, IL-1β, TNF-α, and IFN-γ, either alone or more potently in combination, are clearly detrimental to pancreatic islets resulting in β cell dysfunction, reduced insulin stores, and β cell death (11, 38). When islets isolated from the NOD and C3H mice were incubated with IL-1β (30 U/ml), TNF-α (103 U/ml), and IFN-γ (103 U/ml) for 72 h, significant decreases in viability (29 and 43% of vehicle at 30 and 16 h, respectively) were measured (Fig. 1A). Furthermore, cytokine exposure also had a dramatic effect on islet insulin content reducing the insulin content of NOD islets to 26% of the control value and C3H islets to 39% (Fig. 1B). Previously, we have shown that there is no functional impairment of ALR islets when

**Western blots**

Islets were incubated as above for 30 min either with or without IL-1β (30 U/ml), TNF-α (103 U/ml), and IFN-γ (103 U/ml) for 72 h. Following the cytokine incubation, islets were washed three times in PBS and then aspirated and the slides were washed three times with PBS. Sections were then stained with Sytox Green (Molecular Probes) diluted 1/30,000 with PBS for 1 min, washed with PBS, and then the coverslips mounted. Imaging was performed using a Nikon E800/PC252000 laser scanning confocal microscope (Nikon), and images were acquired with a cooled charged coupled device camera (Photometrics) controlled by the MetaMorph software (Universal Imaging).

**FIGURE 1.** ALR islets resist damage induced by proinflammatory cytokines. Islets isolated from ALR, NOD, and C3H mice were incubated for 72 h in medium without (vehicle) and with IL-1β (30 U/ml), TNF-α (103 U/ml), and IFN-γ (103 U/ml) (cytokines). Cell viability was measured by MTT assay (105 cells/well) (A) and insulin content in islets by RIA (105 islets/dish) (B). Values are means ± SE for four experiments. ***, p < 0.05, ***, p < 0.001 vs vehicle; †, p < 0.001 for vehicle-cytokines vs ALR.
they are treated with this combination of proinflammatory cytokines (8). In Fig. 1, we extend this finding by demonstrating that there is also no decrease in viability and only a small decrease in islet insulin content (83% of control) in ALR islets after cytokine treatment.

**Elevated antioxidant defenses of ALR islets correlate with lowered markers of free radical stress in proinflammatory cytokine-treated ALR islets**

Proinflammatory cytokine treatment of islets isolated from ALR, C3H, and NOD mice resulted in significant increases in medium hydrogen peroxide content; however, the amount of peroxide in the medium from ALR islets was much attenuated compared with both NOD and C3H islets (Fig. 2). The reduction in peroxide production by ALR islets was associated with a significantly higher content of intraislet GSH in ALR than in NOD and C3H islets (Fig. 3A). As we have demonstrated previously (7, 39), ALR islets contain significantly more GSH in comparison to the amount measured in islets from other mouse strains. Treatment of islets with cytokines resulted in significant decreases in GSH in all three mouse strains (Fig. 3A) and concomitant increases in GSSG (Fig. 3B). However, whereas the GSH content of NOD and C3H islets was almost completely ablated after cytokine treatment, the GSH content of cytokine-treated ALR islets remained at the level observed in untreated NOD and C3H islets (Fig. 3A).

Consistent with the significantly higher GSH content in ALR islets and the upkeep of a modest level of GSH in ALR islets after cytokine treatment (Fig. 3A), is the presence of GSR activity in ALR islets (Fig. 4). In previous reports, GSR activity was not measurable in mouse islets with the exception of ALR islets (2, 39). The increase in GSR activity is the result of increased gene expression of GSR (Table I), leading to the presence of GSR protein in ALR islets vs none in controls (data not shown). The result of elevated GSR expression (Table I) correlating with increased GSR activity in ALR islets (Fig. 4) is an increase in the basal level of GSH, as well as the recycling of reduced GSSG to GSH during cytokine treatment (Fig. 3). Furthermore, the increase in GSR activity provides GSH for the detoxification of hydrogen peroxide by GPx. Elevated activity of GPx in ALR islets (Fig. 4) correlates with an increase in Gpx1 mRNA (Table I); therefore, the elimination of cytokine-induced hydrogen peroxide in ALR islets (Fig. 2) is likely due to the elevation in GSR activity in ALR islets. In contrast, the outcome of no GSR activity in NOD islets (Fig. 4) is the elimination of GSH and concomitant increase in GSSG after cytokine treatment (Fig. 3), and the outcome of no GPx activity in NOD islets (Fig. 4) is a failure to detoxify hydrogen peroxide (Fig. 2).

**ALR islets resist cytokine-induced formation of reactive nitrogen intermediates**

Mouse islets deficient in iNOS are resistant to functional inhibition resulting from exposure to combined proinflammatory cytokines (21). This clearly establishes a role for iNOS and NO in cytokine-induced islet dysfunction. To determine whether ALR islets make NO in response to cytokine treatment, we exposed islets from ALR, C3H, and NOD mice to proinflammatory cytokines for 72 h and then harvested the media and assayed for nitrite content. The basal nitrite level was significantly lower in medium from ALR islets than that from NOD or C3H islets (Fig. 5). Upon treatment of islets with cytokines, the level of nitrite in medium from all strains increased significantly; however, nitrite increases were significantly greater from NOD and C3H islets than from ALR islets.

ONOO is a highly reactive radical species produced by the reaction of superoxide with NO (15–17). ONOO is a more potent oxidant and has increased cytotoxic potential compared with either NO or superoxide alone (18–20, 40). The very small increase in H2O2 production from cytokine-treated ALR islets (Fig. 2), coupled with their inability to produce high levels of NO (Fig. 5), should limit the ability of ALR islets to produce ONOO. As expected, the levels of NT, formed by ONOO-induced nitration of tyrosine residues on proteins, did not increase in ALR islets after...
cytokine treatment (Fig. 6A), and the number of NT-positive cells also did not increase (Fig. 6B). In contrast, the levels of NT and the number of NT-positive cells increased significantly in NOD and C3H islets after cytokine treatment. Strikingly, the basal level of NT in ALR islets was ~100-fold lower than levels detected in C3H and NOD islets (Fig. 6A). The findings that ALR islets are strongly resistant to NT formation under both basal and stressful conditions is further evidence of their unusually elevated defense against free radical-mediated damage.

Lack of IKKβ leads to failure of IκB degradation, inhibited p65 NF-κB nuclear translocation, and iNOS synthesis in cytokine-treated ALR islets

As medium nitrite was present at very low levels in cytokine-treated ALR islets, samples were examined to determine whether iNOS was generated in response to cytokine stimulation in ALR islets. Under basal conditions, pancreatic islets did not contain iNOS (Fig. 7, lanes 2, 4, and 6). After 48 h of incubation with IL-1β, TNF-α, and IFN-γ, iNOS was clearly detectable in islets isolated from both C3H (Fig. 7, lane 3) and NOD mice (Fig. 7, lane 5) but still not detectable in ALR islets (Fig. 7, lane 1).

For iNOS to be produced upon cytokine stimulation, the inhibitory subunit of NF-κB, IκB, must be degraded, releasing p65 and p50 NF-κB to translocate to the nucleus and begin transcriptional activity. Significant degradation of IκB can be detected as early as 30 min after exposure (41). Nuclear translocation of p65 NF-κB can be measured as early as 1 h. In untreated NOD islets, there was very little p65 NF-κB in the nucleus (Fig. 8A), but after 1 h of combined cytokine treatment, p65 began to translocate and the p65 (labeled red with PE) and nuclear (labeled with Sytox green) signals began to overlap (Fig. 8). After 16 h of cytokine stimulation, the islet cell nuclei from NOD were positive for p65 (Fig. 8E). In contrast, no translocation was detected in ALR islets at either 1 or 16 h (Fig. 8, D and F). Failure of p65 NF-κB translocation in ALR islets correlated with a failure to degrade the NF-κB inhibitory subunit, IκB (Fig. 9). Unlike NOD islets that exhibited a degradation of IκB after 30 min of cytokine exposure compared with untreated controls, no obvious degradation at this time point was observed in cytokine-treated ALR islets (Fig. 9A). After 72 h of continued exposure to cytokines, IκB concentration in NOD islets had rebounded to a concentration comparable to that in ALR-untreated control islets. However, IκB degradation was now clearly evident in cytokine-treated ALR islets compared with untreated control islets (Fig. 9B). An important finding was that the basal amount of IκB was increased in untreated control ALR islets at both time points (Fig. 9, A and B). The increase in IκB protein was not due to an increase in expression (Fig. 9C). To further explore the basis for the differential degradation rates, we examined IKKβ, a kinase that, once activated, phosphorylates and signals the degradation of IκB. The gene encoding this enzyme on chromosome 8 is contained within the 95% confidence interval for ALR-derived T1D resistance in NOD × ALR outcross/backcross segregation analysis (4). Comparative analysis of IKKβ gene expression by RT-PCR (Fig. 10A) showed normal gene expression in liver of untreated NOD.Rag and ALR mice, as well as in freshly isolated islets from untreated NOD.Rag donors. Surprisingly, no expression was observed in freshly isolated islets from untreated ALR islets.

Table I. Relative differences in gene expression normalized to 18S rRNA

<table>
<thead>
<tr>
<th></th>
<th>Gsr</th>
<th>Gpx1</th>
<th>Ins1/2</th>
</tr>
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<tbody>
<tr>
<td>ALR</td>
<td>155.4 ± 27[^a,b]</td>
<td>111.3 ± 12[^b]</td>
<td>8.8 ± 6.5</td>
</tr>
<tr>
<td>BALB</td>
<td>110.0 ± 7.7</td>
<td>2.6 ± 0.5</td>
<td>9.2 ± 3.4</td>
</tr>
<tr>
<td>B6</td>
<td>5.4 ± 4.3</td>
<td>1.5 ± 0.5</td>
<td>4.8 ± 3.7</td>
</tr>
<tr>
<td>NOD</td>
<td>7.0 ± 3.7</td>
<td>1.9 ± 0.6</td>
<td>8.4 ± 4.7</td>
</tr>
</tbody>
</table>

[^a] Values reported are mean ± SE for three separate islet RNA pools each test in triplicate.
[^b] Denotes significance within a column, p < 0.01.
ALR mice (Fig. 10A). Western blot analysis (Fig. 10B) confirmed this complete lack of IKKβ gene expression in the ALR islets at the protein level. This absence was islet-specific in that Western blot analysis showed the protein was expressed in ALR liver, albeit at lower concentration than observed in NOD liver lysates (Fig. 10B). Absence of a basal level of ALR islet IKKβ expression would certainly explain the altered kinetics of IkB degradation distinguishing cytokine-treated ALR vs NOD islets at the 30-min and 72-h time points (Fig. 9, A and B). This deficiency in IKKβ would be expected to lead to a decrease in signaling velocity through the NF-κB pathway and, likely, allow time for up-regulation of genes conferring protection from oxidative stress.

Discussion

T1D initiation, progression, and culmination in the NOD mouse have logically focused on functional defects in the innate and adaptive arms of this strain’s immune system. When NOD mice are outcrossed with most other inbred strains, either related or unrelated, the F1 hybrid does not develop spontaneous diabetes; however, when F1 mice are reconstituted with an NOD immune
system generated by lethal irradiation and NOD marrow transplantation, F1 β cells are not protected from T cell-mediated destruction (41–43). Accordingly, mouse β cells have been assumed to be passive onlookers to their own destruction in the presence of an activated immune system. The ALR/Lt inbred strain has provided an unusual exception. Despite extensive sharing of H2 alleles, including the diabetogenic class II alleles, as well as important non-MHC alleles in the Idd3 region and elsewhere (3, 4), F1 hybrids generated from NOD × ALR outcross are resistant to T1D developing spontaneously or following irradiation and NOD marrow transplantation (8). This resistance segregated in a genetically dominant fashion and was shown to correlate with an elevated ability to dissipate reactive oxygen species (ROS) systemically (7, 8, 39). Resistance at the isolated islet level correlated both with maintenance of a higher redox potential and the presence of a variant MHC class I allele (3–5, 7, 8, 39).

Considerable evidence indicates that the cytotoxic action of proinflammatory cytokines on rodent pancreatic islets in vitro and in vivo are mediated in part by eliciting free radical generation within β cells themselves (11, 44). The combination of IL-1β, TNF-α, and IFN-γ initiate a variety of gene expressions that activate opposing signal cascades, promoting either destructive or protective responses (45–49). Our results clearly show that these combined cytokines mediate loss of viability in both NOD and C3H but not ALR islets by generating both superoxide and nitrite radicals, as well as the even more potent ONOO radical as evidenced by NT generation and nitrotyrosinylated islet cells. This ALR-unique resistance is unusual as previous reports have demonstrated that islets exhibit a reduced expression and low activity of many antioxidant enzymes (1, 2). Inbred mouse strains show a variety of polymorphisms in genes encoding antioxidant enzymes (50). Given that ALR mice were selected for resistance to alloxan, it is not surprising that they were coselected for elevated constitutive expression of genes encoding multiple antioxidant enzymes. Unusually high strain-specific expression of genes encoding two of the most important antioxidant enzymes, GSR and GPX, confer ALR mice with a systemic ability to dissipate oxygen radical-generated superoxide, even to the extent of suppressing a superoxide burst from activated neutrophils (51).

NF-κB represents a key transcription factor activated by signaling cascades stimulated by combined cytokines, with iNOS induction representing a paradigm downstream target of NF-κB activation. As noted in the Introduction, NO generated by iNOS combines with superoxide to produce ONOO radicals. Nitrosylation of endogenous proteins can generate autoantigens to which NT-reactive CD4+ T cells are not tolerant (52). Cytokine-treated NOD islets, but not ALR islets, up-regulated NF-κB within 1 h of exposure (Fig. 8). Recently published mapping experiments (4) have identified a major ALR-contributed T1D resistance locus on chromosome 8. Data presented in the present study suggest that regulation of the Ikkβ gene mapping to chromosome 8 and encoding IκB may be a component of this ALR-derived resistance (provisionally designated C8-ALR). Our data in Fig. 9 clearly show altered IκB degradation kinetics—data that are consistent with those in Fig. 10 showing that, unlike NOD.Rag islets, ALR islets are not expressing IκB mRNA or protein. Because we observed normal IκB mRNA and protein expression in ALR liver (Fig. 10), either there is a unique islet isoform whose message is not detected by our primer sequences and whose product does not react with our Ab, or ALR islets are unique in maintaining an intracellular redox environment that prevents normal IκB gene expression in the basal state. In marked contrast, NOD dendritic cells (53) and macrophages (54) express very high constitutive NF-κB levels, apparently because of an inability to down-regulate IκB (55). The present data show that this may also be true for NOD islets. Although the kinetics of IκB degradation and thus NF-κB activation elicited by cytokine receptor signaling was slowed in ALR islets (Fig. 9), a small but significant increase in cytokine-mediated hydrogen peroxide generation in ALR islets demonstrated that they were not completely refractory to cytokine-mediated increases in intracellular ROS production (Fig. 2). We hypothesize that the unusual antioxidant defenses exhibited by ALR mice emanate from an increased proton leak from the
mitochondrial electron transport chain. New evidence strongly supportive of our hypothesis is the finding of a novel allelic variant at NADH dehydrogenase subunit 2 (mt-Nd2) encoded by the ALR mitochondrial genome (5). In effect, an altered mitochondrial membrane potential may convey an internal “danger signal” conferring constitutive expression of systemic defenses normally up-regulated in other mouse strains only after application of ROS stress. In human mitochondrial DNA, the same amino acid change of leucine to methionine found in the ALR mtNd2 allele correlated with a decreased incidence of T1D (56). It was suggested that this allelic variant in human mitochondria may render the mitochondria more stress resistant, further limiting free radical production (56). Constitutively up-regulated superoxide dismutase and glutathione recycling enzymes (Table I) coupled with suppressed generation of NO radicals in ALR islets would explain why nitrosylation of cytochrome c and ubiquinone by ONOO−, leading to elevated superoxide/peroxide production, is suppressed systemically in ALR mice (57, 58). In fact, another ALR nuclear gene (on chromosome 3 distal to Idld3 and contributing resistance to T1D development) fully overlapped with an unusual ALR allelic variant, suppressor of superoxide production (Susp). Susp suppressed superoxide production from mitogen-stimulated macrophages and neutrophils (51). Because cytokine treatment affected the intracellular level of both GSH and GSSG, even in ALR islets (Fig. 3), the evidence indicates that ROS are produced in ALR islets in response to combined cytokines but are rapidly detoxified by the antioxidant enzymes GSR and GPX, which are highly expressed in ALR islets (Table I). This rapid dissipation of endogenous ROS, coupled with a suppressed degradation of 1sB, would account for the blunted activation of NF-κB and inhibition of iNOS induction (Fig. 7) and NO production (Fig. 5) in ALR islets (57, 58).

Protein nitration has an adverse effect on mitochondrial function. Analysis of ALR heart mitochondria either following allooxan administration in vivo, or after ONOO exposure in vitro, showed that ALR mitochondrial proteins were protected from NT formation (57). Absence of cytokine-induced NT formation in ALR islets similarly reflects the failure to form NO and either a failure to generate superoxide/peroxide or its rapid dissipation immediately after formation. It is clear from the results presented here that ALR islets make little NO (Fig. 5) and H2O2 (Fig. 2) or rapidly dissipate H2O2 (Figs. 3 and 4). The lack of these two reactive species would reduce iNOS and NO production, coupled with an increased detoxification of the limited ROS that are produced, together preventing nitrosylation of cellular proteins and β cell death.

In conclusion, the ALR genome confers a systemic resistance to free radical stress that is manifested at the pancreatic islet level by a failure of proinflammatory cytokines to activate NF-κB and induce iNOS and NO production, coupled with an increased detoxification of the limited ROS that are produced, together preventing nitrosylation of cellular proteins and β cell death.

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References


