Platelet-type 12-lipoxygenase deletion provokes a compensatory 12/15-lipoxygenase increase that exacerbates oxidative stress in mouse islet \( \beta \) cells

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From the Departments of 1Biochemistry and Molecular Biology, 4Cellular and Integrative Physiology, and 5Pediatrics, Herman B. Wells Center for Pediatric Research, and 6Center for Diabetes and Metabolic Diseases, Indiana University School of Medicine, Indianapolis, Indiana 46202

Edited by Jeffrey E. Pessin

In type 1 diabetes, an autoimmune event increases oxidative stress in islet \( \beta \) cells, giving rise to cellular dysfunction and apoptosis. Lipoxygenases are enzymes that catalyze the oxygenation of polyunsaturated fatty acids that can form lipid metabolites involved in several biological functions, including oxidative stress. 12-Lipoxygenase and 12/15-lipoxygenase are related but distinct enzymes that are expressed in pancreatic islets, but their relative contributions to oxidative stress in these regions are still being elucidated. In this study, we used mice with global genetic deletion of the genes encoding 12-lipoxygenase (arachidonate 12-lipoxygenase, 12S type [Alox12]) or 12/15-lipoxygenase (Alox15) to compare the influence of each gene deletion on \( \beta \) cell function and survival in response to the \( \beta \) cell toxin streptozotocin. Alox12−/− mice exhibited greater impairment in glucose tolerance following streptozotocin exposure than WT mice, whereas Alox15−/− mice were protected against dysglycemia. These changes were accompanied by evidence of islet oxidative stress in Alox12−/− mice and reduced oxidative stress in Alox15−/− mice, consistent with alterations in the expression of the antioxidant response enzymes in islets from these mice. Additionally, islets from Alox12−/− mice displayed a compensatory increase in Alox15 gene expression, and treatment of these mice with the 12/15-lipoxygenase inhibitor ML-351 rescued the dysglycemic phenotype. Collectively, these results indicate that Alox12 loss activates a compensatory increase in Alox15 that sensitizes mouse \( \beta \) cells to oxidative stress.

Type 1 diabetes (T1D) is a debilitating chronic disease caused by autoimmunity-mediated destruction of insulin-producing \( \beta \) cells in pancreatic islets. Because of the loss of these cells, individuals who have T1D must rely on exogenous insulin to maintain glucose homeostasis (1). A complex interplay between genetic and environmental factors leads to the autoimmune attack of the \( \beta \) cells, and recent studies suggest that \( \beta \) cell dysfunction may contribute to neoantigen formation, which instigates autoimmunity (2–4). Oxidative stress resulting from an imbalance in favor of oxidants over antioxidants has been suggested as a primary cause of \( \beta \) cell dysfunction in T1D (5–7). Because of their role in the production and release of insulin, \( \beta \) cells generate endogenous reactive species such as hydrogen peroxide, hydroxyl radicals, and peroxyxinitrites. Paradoxically, \( \beta \) cells possess relatively low antioxidative capacity because of low expression of antioxidant enzymes (8, 9). Interventions that augment antioxidant capacity in mouse models of T1D protect against immunity-mediated \( \beta \) cell destruction (10, 11). Hence, uncovering mechanisms that influence the oxidative stress response in \( \beta \) cells would provide new targets for the treatment of this disease.

Lipoxygenases (LOXs) are lipid-processing enzymes that catalyze the oxygenation of polyunsaturated fatty acids (12). Depending on the specific substrate, lipoxygenases produce a variety of different lipid metabolites that regulate a host of biological functions and disease states (13–15). In mice, the gene Alox15 encodes for “leukocyte-type” 12-lipoxygenase (known as 12-LOX), which catalyzes oxygenation of lipids at carbon 12 or 15 (16, 17). The related gene Alox12 encodes “platelet-type” 12-lipoxygenase (known as 12-LOX), which oxygenates lipids at carbon 12. Both enzymes catalyze the oxygenation of the polyunsaturated fatty acid arachidonic acid, with 12-15-LOX forming 12- and 15-hydroxyeicosatetraenoic acid (HETE) in a 6:1 ratio, whereas 12-LOX forms only 12-HETE. The activity of 12-15-LOX and its major lipid metabolite, 12-HETE, have been linked to the pathogenesis of T1D. Mice harboring whole-body knockout of Alox15 show protection from low-dose streptozotocin (STZ)–induced diabetes (18). Likewise, non-obese diabetic mice with whole-body knockout of Alox15 also show...
Deletion of 12-LOX exacerbates β cell dysfunction, whereas deletion of Alox15 is protective.

Deletion of 12-LOX exacerbates STZ-induced diabetes, whereas deletion of Alox15 is protective.

We sought to assess the metabolic effects of whole-body deletion of the genes encoding 12/15-LOX and 12-LOX (Alox15 and Alox12, respectively) in mice on the C57BL6/J background. As shown in Fig. S1, A–D, Alox15−/−, Alox12−/−, and their WT control littermate mice at 8 weeks of age exhibited no differences in body weight, glucose tolerance (by intraperitoneal glucose tolerance tests (GTTs)), or β cell mass (by histomorphometric analysis of immunostained pancreata). These results are consistent with prior findings (22–24) that loss of Alox12 or Alox15 does not appear to affect the normal development of β cells or whole-body glucose homeostasis.

To assess whether loss of Alox15 and Alox12 negatively or positively affects β cell function during the development of diabetes, we leveraged the multiple low-dose STZ model (55 mg/kg body weight STZ intraperitoneally daily for 5 days) to induce diabetes. In this β cell toxicity model, mice develop a T1D-like phenotype with local islet inflammation and consequent hyperglycemia over 4 weeks (24–26). As expected, WT mice developed overt diabetes (blood glucose ≥ 300 mg/dl) within 14 days following STZ injections (Fig. 1A, closed circles). Consistent with data published previously (19), Alox15−/− mice were protected from overt diabetes following STZ (Fig. 1A, gray circles). In striking contrast, Alox12−/− mice exhibited more severe hyperglycemia than WT mice by 14 days following STZ and continuing through the conclusion of the study at 28 days (Fig. 1A, open circles). GTTs performed 4 days following STZ revealed that Alox12−/− mice were significantly more glucose-intolerant than WT and Alox15−/− mice, whereas Alox15−/− mice showed protection from STZ-induced glucose intolerance compared with WT mice (Fig. 1, B and C).

We next evaluated β cell mass in STZ-treated mice. As shown in Fig. 1, D and E, STZ-treated Alox12−/− mice exhibited a significant 1.8-fold reduction in β cell mass compared with STZ-treated WT mice. In contrast, β cell mass was more than 2-fold higher in STZ-treated Alox15−/− mice compared with STZ-treated WT mice (Fig. 1, D and E). Together, these data suggest that loss of Alox12 exacerbates inflammation-induced β cell dysfunction, whereas loss of Alox15 is protective in this setting.

Deletion of Alox12 exacerbates inflammation-induced oxidative stress in β cells

12-HETE, a lipid product of 12-LOX and 12/15-LOX, is linked to oxidative stress in islets (27). We therefore asked whether oxidative stress in β cells differed in WT, Alox15−/−,
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and Alox12−/− mice after STZ treatment. We examined oxidative stress in tissue sections from STZ-treated control and knockout mice by analysis of islet 4-hydroxynoneal (4-HNE) (28, 29) by immunofluorescence. STZ-treated Alox15−/− mice exhibited ∼2-fold reduced 4-HNE immunostaining intensity compared with STZ-treated WT mice (Fig. 2, A and B). However, in agreement with their exacerbated diabetic phenotype, Alox12−/− mice exhibited a significant 1.4-fold increase in 4-HNE immunostaining intensity compared with STZ-treated WT mice (Fig. 2, A and B). To determine whether the opposing effects on oxidative stress observed between Alox15−/− and Alox12−/− mice could be due to differential production of antioxidant enzymes, we co-immunostained pancreatic sections from STZ-treated WT, Alox15−/−, and Alox12−/− mice following STZ-induced diabetes. We observed significant increases in cell immunostaining intensities of both GPX1 and CAT in Alox15−/− mice compared with both WT and Alox12−/− mice (Fig. 2, C–F). Conversely, Alox12−/− mice showed a significant decrease in β cell GPX1 immunostaining compared with WT and Alox15−/− mice (Fig. 2, C and D). These results suggest that loss of Alox15 and Alox12 result in changes in antioxidant protein levels in β cells that may explain their observed effects on β cell oxidative stress.
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Alox12 deletion exacerbates reactive oxygen species accumulation in β cells

To determine whether the increased oxidative stress was a result of elevated reactive oxygen species (ROS) generation in the islet, we employed the redox biosensor redox-sensitive GFP (roGFPR) (30, 31) to measure ROS accumulation dynamically. Islets were transduced with an adenovirus harboring roGFPR and then treated for 16 h with a proinflammatory cytokine mixture (IL1-β, TNFα, and IFN-γ) to approximate inflammation-induced oxidative stress. As shown in the representative ratiometric image in Fig. 3A and as quantified in Fig. 3B, islets from WT mice exhibited an increased redox ratio after cytokine treatment, signifying that cytokines increased ROS. In support of our observations in vivo, cytokine-treated Alox15−/− islets exhibited significantly less ROS elevation in response to cytokines compared with WT islets. In contrast, cytokines elicited significantly increased ROS in Alox12−/− islets compared with WT islets (Fig. 3, A and B). Collectively, these results suggest that deletion of Alox15 protects against ROS accumulation, whereas deletion of Alox12 promotes enhanced oxidative stress.

Alox12 deletion decreases circulating eicosanoid levels in mice

The products of LOXs are biologically active lipid metabolites, and loss of LOXs is expected to alter the levels or ratios of these metabolites, which may account for the effects observed in our mice. To determine the changes in eicosanoid profiles of Alox12−/− and Alox15−/− mice, we performed LC-MS/MS analysis of key eicosanoids in the circulation of mice. There was no statistically significant difference in circulating levels of 5-HETE, 15-HETE, 13-HODE, 17-HDHA, and LTB4 in Alox12−/− and Alox15−/− mice compared with WT mice (Table 1). Levels of 12-HEPE were significantly increased in Alox15−/− mice compared with controls. There were expected reductions in 12-HETE, which reached statistical significance in Alox12−/− mice (p = 0.02) and approached significance in Alox15−/− mice (p = 0.06) compared with WT controls. These data confirm that our knockout mice exhibited reductions in the major 12-LOX and 12/15-LOX metabolite in the circulation.

Alox12 deletion increases STZ-induced oxidative damage through reciprocal up-regulation of Alox15

We next asked how loss of Alox12 could render mice more sensitive to STZ-induced diabetes. Because Alox15 and Alox12 are functionally related, we analyzed the expression of each gene in both Alox15−/− and Alox12−/− islets and compared them with expression levels in WT controls. Quantitative PCR analysis of Alox12 mRNA in WT, Alox15−/−, and Alox12−/− islets show the expected decrease in Alox12−/− islets and no significant change in Alox15−/− islets (Fig. 4A). Strikingly, Alox12−/− mice exhibited a 28-fold increase in Alox15 mRNA expression compared with the WT (Fig. 4B), suggesting that loss of Alox12 leads to a compensatory up-regulation of Alox15 but not vice versa. We hypothesized that the striking reductions in 12-HETE observed in Alox12−/− mice (Table 1) might cause a compensatory increase in Alox15 in this setting, implying that 12-HETE levels might inversely regulate Alox15. To test this hypothesis, we incubated Min6 β cells overnight with 100 nM 12-HETE and performed gene expression analysis by quantitative PCR. Treatment with 12-HETE caused a significant reduction in Alox15 expression compared with the control (Fig. 4C), a result that supports our hypothesis. We also sought to determine the effect of 12-HETE on the expression of antioxidant genes. Incubation of Min6 cells with 12-HETE caused a significant decrease in Gpx1 expression (Fig. 4D). The expression of Catalase was increased following 12-HETE incubation but was not statistically significant (Fig. 4E).

To determine whether the increase in Alox15 expression contributes to the increased sensitivity of Alox12−/− mice to STZ, we employed the 12/15-LOX–specific small-molecule inhibitor ML351 (32). Alox12−/− mice treated with ML351 were protected from STZ-induced glucose intolerance compared with vehicle-treated Alox12−/− mice (Fig. 4, F and G). These results suggest that 12/15-LOX levels in Alox12−/− mice augment the sensitivity of these mice to STZ-induced dysfunction.

Discussion

LOXs form a family of enzymes that catalyze the oxygenation of cellular polyunsaturated fatty acids to form lipid inflamma-
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Table 1
Alox12 deletion decreases circulating eicosanoid levels in mice

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<th>WT</th>
<th>Alox15&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Alox12&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<td>p value vs. WT</td>
<td>Mean (ng/ml) ± S.E.</td>
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<tr>
<td>5-HETE</td>
<td>2.88 ± 0.48</td>
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<td>1.75 ± 0.99</td>
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<tr>
<td>12-HETE</td>
<td>3433 ± 1143</td>
<td>-</td>
<td>1266 ± 234</td>
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<tr>
<td>15-HETE</td>
<td>7.85 ± 2.53</td>
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<td>3.55 ± 1.60</td>
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<td>12-HEPE</td>
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<tr>
<td>13-HODE</td>
<td>77.33 ± 24.93</td>
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<td>515.0 ± 237.6</td>
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<td>LTB4</td>
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Figure 4. Alox12 deletion increases STZ-induced oxidative damage through reciprocal up-regulation of Alox15.

A, Alox12 gene expression in islets from WT, Alox12<sup>−/−</sup>, and Alox15<sup>−/−</sup> mice. B, Alox15 gene expression in islets from WT, Alox12<sup>−/−</sup>, and Alox15<sup>−/−</sup> mice. C, Alox15 gene expression in Min6 cells following overnight incubation with vehicle or 12-HETE. D, Gpx1 gene expression in Min6 cells following overnight incubation with vehicle or 12-HETE. E, catalase gene expression in Min6 cells following overnight incubation with vehicle or 12-HETE. F, analysis of glucose tolerance (GTT) in vehicle- or ML351-treated Alox12<sup>−/−</sup> mice 4 days following the STZ regime. G, area under the curve (AUC) analysis of GTTs. *p < 0.05. **p < 0.01.
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![Figure 5. Proposed model of Alox12 versus Alox15. Deletion of 12/15-LOX(Alox15) leads to a decrease in 12-HETE levels, an increase in 12-HEPE, augmentation of the antioxidant response in β cells during oxidative stress, and increased survival. Deletion of 12-LOX(Alox12), on the other hand, causes a compensatory increase in 12/15-LOX(Alox15) expression, loss of 12-HEPE production, inhibition of the antioxidant response, and β cell dysfunction.](image-url)

has been shown to be protective in both STZ and nonobese diabetic mouse models of T1D, effects that have been ascribed to reductions in the levels of 12-HETE (18–20, 33). The proinflammatory actions of 12-HETE may be due to its properties as a lipid peroxide and via its interactions with its receptor Gpr31 (34, 35). Although 12-HETE is also produced by 12-LOX, a role of the Alox12 gene in β cell oxidative stress during diabetes pathogenesis has not been interrogated. In this study, we interrogated the roles of 12/15-LOX and 12-LOX in the development of oxidative stress in pancreatic islets by studying their respective gene knockouts (Alox15 and Alox12) in the context of T1D models in vivo and in vitro. Although our data confirm a pro-diabetogenic role of Alox15, we found a protective effect for Alox12. Our data demonstrate that loss of Alox12 leads to compensatory hyperactivity of 12/15-LOX encoded by Alox15 (Fig. 5).

We observed that knockout of either Alox12 or Alox15 did not appear to affect β cell mass or glucose homeostasis, findings that are in concordance with prior studies that showed no gross phenotype in unchallenged animals (18, 22). However, a striking and discordant phenotype between the two knockouts was observed when islets of these animals were subjected to proinflammatory stress using the multiple low-dose STZ protocol. STZ is selectively taken up by β cells through the Glut2 transporter and leads to DNA alkylation and eventual cell death (36). Low doses of STZ trigger low-frequency β cell killing, which is exacerbated by macrophage influx, proinflammatory cytokine release, and oxidative stress (37). Under these conditions, prior studies have shown that Alox15−/− mice are protected from β cell destruction and hyperglycemia (18). Our results with Alox15−/− mice confirm these prior studies but add the vital observation that protection from β cell dysfunction may be caused by reductions in oxidative stress, as judged by reduced immunostaining for 4-HNE accompanied by enhanced immuno-nostaining for the antioxidant enzymes GPX1 and CAT. In contrast, we show that Alox12−/− mice exhibit exacerbated hyperglycemia compared with WT controls, with enhanced immunostaining for 4-HNE in β cells accompanied by reduced immunostaining for GPX1 and CAT. Our studies provide the first documented evidence that the enzymes encoded by Alox15 and Alox12 display apparently opposing roles in β cell oxidative stress, notwithstanding that both enzymes produce identical major products (12-HETE).

At least two possibilities might be invoked to account for the disparate phenotypes of Alox15−/− mice and Alox12−/− mice. First, it is possible that the products each enzyme produces and/or their relative ratios can affect the net production of ROS. In this regard, although arachidonic acid and its metabolites are important in cytokine-induced β cell dysfunction (38), it is not the only substrate for these enzymes, and the preferential utilization of other key substrates, such as dihomo-γ-linolenic acid or eicosapentaenoic acid (EPA), form products that may be protective (13, 39). Indeed, we observed an increase in circulating levels of the EPA-derived metabolite 12-HEPE in Alox15−/− mice that was undetected in the Alox12−/− mice. This finding raises the possibility that loss of Alox15 leads to preferential use of EPA over arachidonic acid as a product. This switch to EPA metabolism has been shown to be protective of β cell function (40). Second, it is possible that the catalytic similarities between the two enzymes might allow one enzyme to compensate to produce the products of the other. We show here that, although no up-regulation of Alox12 was observed in Alox15−/− mice, there was a nearly 30-fold increase in Alox15 levels in Alox12−/− mice. The near-reversal of the diabetogenic phenotype of Alox12−/− mice through concurrent pharmacologic inhibition of 12/15-LOX (with ML351) supports the notion that 12/15-LOX activity contributes to the phenotype of these animals. We recognize, however, that we cannot rule out a contribution resulting from the loss of potentially protective lipid products emanating from 12-LOX activity in Alox12−/− mice. Based on the lipidomics analysis, we observed a decrease in 12-HETE in the Alox12−/− mice. This suggests that the observed detrimental effects observed because of loss of Alox12−/− is not caused by an increase in 12-HETE levels. Additionally, we show that 12-HETE inhibits the expression of Alox15 in Min6 cells. Taken together, the observed decrease in 12-HETE in Alox12−/− mice could then lead to the observed up-regulation of Alox15.

In conclusion, our data suggest that Alox15 and Alox12 play disparate roles in the pathogenesis of β cell oxidative stress and dysfunction. The observation that Alox15 levels are elevated in Alox12−/− islets also highlights a deficiency in our understanding of the regulation of the genes encoding LOX enzymes. As inhibitors of LOX enzymes begin to gain traction for disease modification (32, 41), it will be especially relevant to understand how inhibition of specific enzymes might influence the expression and activities of other related LOXs. Future studies unraveling the effect of complete loss versus inhibition of a lipooxygenase enzyme and possible compensatory effects are thus warranted.
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Materials and methods

Cells, animals, and procedures

All experiments involving mice were performed with approval from the Indiana University Institutional Animal Care and Use Committee. B6.129S2- Alox12tm1Fun/J (Alox12−/−) and B6.129S2- Alox15tm1Fun/J (Alox15−/−) mice were purchased from The Jackson Laboratory and maintained in the Indiana University School of Medicine animal facilities. WT mice were littermates from both Alox15 and Alox12 litters. All mice were maintained under pathogen-free conditions. Male mice were injected intraperitoneally daily for 5 consecutive days with saline or streptozotocin at 55 mg/kg/day at 8 weeks of age. A cohort of mice received the 12/15-LOX inhibitor ML351 at 10 ng/kg body weight for 5 days prior, during, and 5 days post-STZ treatment (32). Glucose measurements and intraperitoneal GTTs at 2 g/kg body weight of glucose were performed as described previously (21). At the end of the respective studies, mice were euthanized, serum was collected, and pancreata were harvested and fixed for analysis. Alternatively, mouse islets from both male and female mice were isolated from collagenase-perfused pancreata and cultured in RPMI medium as described previously (42, 43). Islets were hand-picked and allowed to recover overnight in complete medium before experimentation. The mouse insulinoma cell line MIN6 was maintained in high-glucose DMEM with 15% FBS and 1% penicillin/streptomycin supplemented with 10 mM HEPES and sodium pyruvate and treated with 100 nM 12-HETE for 24 h, followed by RNA isolation using the RNeasy Mini Kit (Qiagen).

Immunohistochemistry, immunofluorescence, and β cell mass

Pancreata were fixed in 4% paraformaldehyde, sectioned, and immunostained for insulin as described previously (44). Pancreata were stained by immunofluorescence using the following primary antibodies: anti-4-HNE antibody (Ab464545, Abcam, 1:100), anti-GPX1 (Santa Cruz), anti-CAT (Santa Cruz), and anti-insulin antibody (A0564, Dako, 1:500). Alexa Fluor 568 donkey anti-rabbit antibody and Alexa Fluor 488 donkey anti-goat pig antibody were used as secondary antibodies (Invitrogen). Images were acquired using a Zeiss LSM 700 or LSM 800 confocal microscope. 4-HNE immunostainings were quantified by measuring pixel density per insulin-positive cell. β Cell mass was calculated as described previously using at least three pancreas sections 70 μm apart from five pancreata per group (45).

Measurement of β cell redox state

Mouse islets were isolated as described above. After overnight recovery, islets were briefly washed with PBS and dispersed by incubation with Accutase (STEMCELL Technologies) for 1 min at 37 °C. Accutase was then inactivated, and islets were resuspended in complete islet medium containing serum. Islets were then transduced with the roGFP2 adenovirus for 6 h in complete islet medium. Following transduction, islets were then transferred to virus-free islet medium containing a vehicle or proinflammatory cytokine mixture (5 ng/ml IL-1β, 10 ng/ml TNFα, and 100 ng/ml IFN-γ) for 16 h. Islets were then imaged on a Zeiss LSM 700 confocal microscope. The biosensor was sequentially excited with 405- and 488-nm excitation, and GFP fluorescence was collected and ratioed.

Eicosanoid analysis

Mouse serum collected from WT, Alox12−/−, and Alox15−/− mice was analyzed by the Washington University Metabolomics Core for profiling of eicosanoids (5-HETE, 12-HETE, 8-HETE, 15-HETE, 12-HEPE, 13-HODE, 17-HDHA, and LTB4). Eicosanoids were extracted from 50 μl of serum with 200 μl of methanol containing 2 ng each of deuterated 5-HETE-d9, 13-HODE-d9, and LTB4-d9 as the internal standards. The supernatant was reconstituted with 250 μl of water for MS analyses. Four-point to seven-point calibration standards of all eicosanoids containing the deuterated internal standards were prepared for absolute quantification. The sample analysis was performed with a Shimadzu 20AD HPLC system and a SIL-20AC autosampler coupled to a tandem mass spectrometer (API-6500+, Applied Biosystems) operated in multiple reaction monitoring mode. The negative ion ESI mode was used for detection of these eicosanoids. The plasma extracts were injected in duplicate for data averaging. Data processing was conducted with Analyst 1.6.3 (Applied Biosystems).

Real-time RT-PCR

Reverse-transcribed islet and MIN6 RNA was analyzed by real-time PCR using SYBR Green or TaqMan technology. Primers included Gpx1, Cat, Alox15, and Alox12 (Qiagen). All samples were corrected for input RNA by normalizing to the Actb message. All data represent the average of independent determinations from at least three separate mice.

Statistical analysis

All experiments were completed in at least biological triplicates. All data are presented as the mean ± S.E. One-way analysis of variance (with Holm–Sidak’s post test) was used for comparisons involving more than two conditions, two-way analysis of variance was used for comparisons with multiple time points, and two-tailed Student’s t test was used for comparisons involving two conditions. Prism 8 software (GraphPad) was used for all statistical analyses. Statistical significance was assumed at p < 0.05.


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