Integration of Pro-Inflammatory Cytokines, 12-Lipoxygenase and NOX-1 in Pancreatic Islet Beta Cell Dysfunction.

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ABSTRACT

Elevated cellular reactive species, which can be produced by diabetic serum conditions such as increased inflammatory cytokines, contribute to islet beta cell dysfunction and cell death. Cellular pathways that result in beta cell oxidative stress are poorly resolved. In this study, stimulation of human donor islets, primary mouse islets or homogeneous beta cell lines with a cocktail of inflammatory cytokines (TNFα, IL-1β, INFγ) significantly ($p<0.05$) induced NADPH oxidase-1 (NOX-1) gene expression. This pro-inflammatory cytokine cocktail concomitantly induced loss of islet glucose stimulated insulin response ($p<0.05$), elevated expression of MCP-1 ($p<0.01$), increased cellular reactive oxygen species (ROS) and induced cell death. Inhibitors of NADPH oxidase, apocynin and diphenylene iodonium, blocked ROS generation ($p<0.01$) and induction of MCP-1 ($p<0.05$) by pro-inflammatory cytokines in beta cells. It has previously been reported that pro-inflammatory cytokine stimulation induces 12-lipoxygenase (12-LO) expression in human islets. 12-hydroxyeicosatetraenoic acid (12-HETE), a product of 12-LO activity, stimulated NOX-1 expression in human islets ($p<0.05$). Additionally, a new selective inhibitor of 12-LO blocked pro-inflammatory cytokine-induction of NOX-1 in INS-1 beta cells ($p<0.01$). Inhibition NOX-1 was not seen with a structural analogue with no 12-LO inhibitory activity. Importantly, islets from human type 2 diabetic donors have an elevated expression of NOX-1 ($p<0.05$). This study reports the initial description of an integrated pathway in beta cells that links pro-inflammatory-induced beta cell dysfunction with 12-lipoxygenase and NADPH oxidase activation. Inhibitors of this pathway may provide a new therapeutic strategy for diabetes.

KEYWORDS:

NADPH Oxidase, 12-Lipoxygenase, Cytokines, Diabetes, Islet beta cell, Inhibitors.

1. INTRODUCTION

Oxidative Stress in the pancreatic beta cell is recognized as a pathogenic step associated with a loss of beta cell function. Pancreatic beta cells express a lower activity of free-radical detoxifying enzymes (eg, catalase, superoxide dismutase, glutathione peroxidase) when compared to other tissues (Grankvist, Marklund et al. 1981; Lenzen, Drinkgern et al. 1996; Tiedge, Lortz et al. 1997; Modak, Datar et al. 2007). Beta cells are also inefficient in rectifying oxidative damage to DNA (Modak, Parab et al. 2009). Thus, under conditions of sustained activation of intracellular reactive species, islets are readily overwhelmed and undergo oxidative stress (Lenzen 2008). Under oxidative stress conditions, the elevated reactive oxygen species (ROS), in addition to oxidizing proteins, lipids and DNA, also activate stress-sensitive second messengers such as p38MAPK, JNK (Purves, Middlemas et al. 2001) and PKC (Koya and King 1998). While a transient increase in ROS generation is a required second messenger for glucose stimulated insulin secretion in the beta cell (Goldstein, Mahadev et al. 2005; Pi, Bai et al. 2007; Morgan, Rebelato et al. 2009; Newsholme, Morgan et al. 2009), chronic activation of ROS is destructive to the function and survival of the beta cell.

Serum conditions associated with the diabetic state, increased pro-inflammatory cytokines, high free fatty acids (FFA) and elevated glucose levels are all potent inducers of high levels of cellular ROS (Janciauskiene and Ahren 2000; Oliveira, Verlengia et al. 2003; Cunningham, McClenaghan et al. 2005; Inoguchi and Nawata 2005; Nakayama,

Inoguchi et al. 2005; Uchizono, Takeya et al. 2006; Morgan, Oliveira-Emilio et al. 2007; Michalska, Wolf et al. 2010). Chronic elevation of pro-inflammatory cytokines is an established feature of type 1 diabetes (Eizirik and Mandrup-Poulsen 2001; Jorns, Gunther et al. 2005), and in recent studies low-grade chronic inflammation and increase in serum pro-inflammatory cytokines is recognized as a key feature of type 2 diabetes (Catalan, Gomez-Ambrosi et al. 2007; Steinberg 2007; Tilg and Moschen 2008; Al-Maskari, Al-Shukaili et al. 2010; Igoillo-Esteve, Marselli et al. 2010; Kang, Song et al. 2010; Su, Pei et al. 2010). Within the beta cell, cellular sources of ROS include induced mitochondrial stress (reviewed (Newsholme, Haber et al. 2007)), induced endoplasmic reticulum stress (reviewed (Volchuk and Ron 2010)) and potentially NADPH oxidase activation.

NADPH oxidases are proteins that transfer electrons across biological membranes. Their function is the generation of ROS, superoxide and hydrogen peroxide (H_20_2). The phagocyte NADPH oxidase was the first identified example of an enzyme system where ROS generation was the primary function rather than a byproduct, as seen in mitochondria and other cell components. Activation of phagocyte NADPH oxidase occurs through a complex series of protein interactions. Genome sequencing has subsequently identified a family of NOX proteins that form distinct NADPH oxidase complexes. NOX-1 is one of five homologues of the core catalytic component subunit of phagocyte NADPH oxidase (reviewed (Bedard and Krause 2007)). NOX isoforms and subunits of the functional complex have been identified in pancreatic beta cells supporting a role of NADPH oxidase in beta cell function/dysfunction (Cheng, Cao et al.

2001; Uchizono, Takeya et al. 2006; Kowluru 2010). Global inhibition of NADPH oxidase conferred protection to beta cell dysfunction induced by cytokines or fatty acids (Michalska, Wolf et al. 2010). Most recently we have shown that decreased NOX-1 expression is associated with beta cell protection in a transgenic model (Chang, Weaver et al. 2010).

In non-beta cell systems, activators of NOX activity include 12-lipoxygenase (12-LO), a lipid metabolizing enzyme. 12-LO converts arachidonic acid to lipid mediators including 12-hydroxyeicosatetraenoic acid (12-HETE). Platelet oxidation, generated by NADPH oxidase, is downstream of 12-LO (Nardi, Feinmark et al. 2004). Exposure of cultured neurons to methylisothiazolinone induced 12-LO which increased ERK activation and NADPH oxidase activation (Du, McLaughlin et al. 2002), linking 12-LO to ERK activation of NOX. Further, induction of monocyte chemotactic protein (MCP)-1 in peritoneal macrophages by 12-S-HETE (a product of 12-LO activity) was blocked by inhibitors of NADPH oxidase activity (apocynin and diphenyleneiodonium chloride) (Wen, Gu et al. 2008). In pancreatic islets, MCP-1 expression is inversely correlated with islet graft function (Marzorati, Antonioli et al. 2006). Additionally, proliferation and migration of colon adenocarcinoma cells is mediated by NOX-1 (de Carvalho, Sadok et al. 2008; Sadok, Bourgarel-Rey et al. 2008) that is stimulated by 12-LO or arachidonic acid (the substrate for 12-LO activity); inhibition of 12-LO decreased arachidonic acid induced NOX-1 expression and cell migration. However an interaction between 12-LO and NOX-1 has not been described in pancreatic beta cells.

12-Lipoxygenase activity has been linked with the development in T1DM. 12-LO knock out mice were resistant to the induction of diabetes by low dose streptozotocin (Bleich, Chen et al. 1999) and the 12-LO knock out mice lacked cytokine-induced conversion of arachidonic acid to 12-HETE, implying that 12-HETE generation was cytotoxic to beta cells (Bleich, Chen et al. 1999). The role of 12-LO as a key mediator in the development of autoimmune diabetes is supported by studies of 12-LO deletion in the type 1 diabetes (T1DM) mouse model, NOD mice. Knock out of leukocyte 12-LO in NOD mice resulted in a significant reduction (2.5% *vs* >60%) in the development of diabetes (McDuffie, Maybee et al. 2008). A direct role of pro-inflammatory cytokines in activating 12-LO activity is supported by cytokine-induced production of 12-HETE in both islets and beta-cell lines (Bleich, Chen et al. 1995; Chen, Yang et al. 2005). Addition of 12-HETE to human islets decreased islet viability and function (Ma, Nunemaker et al. 2010).

In this study, we describe activation of NOX-1 by pro-inflammatory cytokines in islets and beta cell lines. This upregulation in NOX-1 is linked to 12-LO activity and reproduced in islets from diabetic donors. The data described integrate pro-inflammatory cytokine induced beta cell dysfunction with 12-LO activation and NOX-1-induced ROS in a unifying pathway.

2. MATERIALS AND METHODS

2.1 Institutional approvals were obtained for all procedures and resources described.

2.2 *Reagents and Cell lines*: Human donor islets were obtained from integrated islet distribution project (http://iidp.coh.org) and cultured in CMRL media (Mediatech, Manassas, VA). Mouse islets were freshly isolated by common bile duct cannulation and collagenase digestion. Islets were hand picked prior to use. Beta cell lines, INS-1 and βTC3 were cultured as described (Scharfmann, Tazi et al. 1993). Active inhibitors of 12-lipoxygenase and inactive structural analogues were kindly provided by NIH Chemical Genomics Center, Bethesda, MD. Other reagents were commercially sourced, human and mouse cytokines (R&D Systems, Minneapolis, MN), Apocynin, Diphenylene Iodonium Chloride (DPI), (Sigma Aldrich, St Louis, MO), 12-hydroxyeicosatetraenoic acid (12-HETE, Enzo Life Science, Farmingdale, NY).

2.3 *Cell stimulation*: Cells or islets were treated with (TNFα 1ng/ml, IL-1β 0.5ng/ml, IFNγ 10ng/ml) individually or in combination (triple cytokines) for 4 or 24 hours. Recovered cell pellets were analyzed for gene expression by real-time quantitative PCR as previously described (Ma, Nunemaker et al. 2010). Oxidative stress PCR array was performed following manufacturers instructions (SABiosceinces, Valencia, CA). All reactions were done in triplicate. Primer sequences used were; Human NOX-1 forward 5'-CAC AAG AAA AAT CCT TGG GTC AA-3'; Human NOX-1 reverse 5'-GAC AGC AGA TTG CGA CAC ACA-3'; Nox1 forward 5'-CGC TCC CAG CAG AAG GTC GTG ATT ACC AAG G-3'; Nox1 reverse 5'-GGA GTG ACC CCA ATC CCT GCC CCA ACC A-3'; MCP-1 forward, 5'-CTT CTG GGC CTG CTG TTC A-3'; MCP-1 reverse 5'-CCA GCC TAC TCA TTG GGA TCA-3'; Actin forward, 5'-AGG TCA TCA CTA TTG GCA ACG A-3'; Actin reverse, 5'-CAC TTC ATG ATG GAT TGA ATG TAG TT-3'; GAPDH

forward 5'-TCA CCA CCA TGG AG-3'; GAPDH reverse 5'-GCT AAG CAG TTG GT-3'. Taqman primers were used for caspase 3, human GAPDH and human 12-Lipoxygenase (Applied Biosystems, Carlsbad, CA).

2.4 *Detection of Reactive Species and Apoptosis*: Treated INS-1 cells were washed in PBS and placed in PBS containing 10μM 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (DCF-DA, Invitrogen, Carlsbad, CA) for 30 mins at 37ºC. Following a PBS wash and re-incubation in PBS for 1 hr at 37ºC, fluorescence was measured on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA); emission wavelength 480 nm; excitation wavelength 530 nm. Islet apoptosis was screened microscopically. Islets were washed in PBS and incubated in ice cold PBS containing 1μg/ml Propidium Iodide and 0.1μM YO-PRO-1 (Invitrogen) for 30 min. Fluorescent signal was determined on an AxioObserver microscope (Carl Zeiss Inc.) using defined settings. Quantitation of fluorescent signal was achieved with Image J (rsbweb.nih.gov/ij/).

2.5 *Data and analysis*: All experiments were performed a minimum of three times. Data shown are mean ± SEM. Data was analyzed using Prism 4.0 (GraphPad Software, Inc., La Jolla, CA). Significance was calculated using student two-tail t-tests with 95% confidence and defined as $p<0.05$.

3. RESULTS AND DISCUSSION

Treatment of human donor islets with a cocktail of pro-inflammatory cytokines (TNFα, IL-1β, IFNγ) for 24 hours induced cell death as detected microscopically (Supplemental Figure 1A/B) and validated by increased gene expression of the apoptosis gene Caspase-3 (Supplemental Figure 1C). Disruption of islet function by pro-inflammatory cytokines (PICs) was further confirmed by a loss of the glucose-stimulated-insulin-response (Supplemental Figure 1D). PCR array analyses were performed with focus on oxidative stress pathways to determine intracellular pathways that are initiated by PICs leading to beta cell dysfunction (data not shown). An increase in expression of NADPH oxidase NOX-1 was identified and subsequently validated in quantitative real time PCR analysis (Figure 1). Stimulation with a cocktail of pro-inflammatory cytokines (TNFα, IL-1β and IFNγ), at relevant concentrations, resulted in a significant ($p<0.05$) increase in expression for NOX-1. A significant ($p<0.05$) fold elevation in NOX-1 expression was observed in human donor islets, in freshly isolated mouse islets and also in homogeneous beta cell lines, mouse βTC3 cells and rat INS-1 cells following pro-inflammatory cytokine stimulation. Other components of the functional NOX-1 complex have been described in beta cells (Cheng, Cao et al. 2001; Uchizono, Takeya et al. 2006; Kowluru 2010). Thus, elevated NOX-1 expression by PICs could result in a sustained increase in ROS generated via the NADPH oxidase complex. The induction of NOX-1 by triple pro-inflammatory cytokine combination was not reproduced by individual cytokine stimulation (Supplemental Figure 1E) suggesting an interaction between the triple cytokine stimulation occurs in beta cells to induce NOX-1 expression. Triple cytokine stimulation also induced markers of beta cell dysfunction, such as apoptotic markers, loss of glucose-stimulated insulin response and expression of MCP-

1, to levels that are not reproduced by individual cytokine stimulation (JW and DATF data not shown).

Elevated expression of MCP-1 is a recognized marker of beta cell dysfunction. Indeed, for human islet transplantation protocols, MCP-1 expression is inversely correlated with islet graft function (Marzorati, Antonioli et al. 2006). We therefore used MCP-1 expression as a marker of PIC-induced islet dysfunction to determine the importance of the NADPH oxidase in beta cell damage. Highly selective inhibitors of NOX isoforms and NADPH oxidase are not available; however, extensive literature on the use of apocynin and diphenylene iodonium chloride (DPI) as inhibitors of NADPH oxidase are published (Lambeth, Krause et al. 2008). Stimulation of the INS-1 beta cell line with triple pro-inflammatory cytokines produced a marked elevation in MCP-1 expression, consistent with induction of beta cell dysfunction (Figure 2). Treatment with apocynin or DPI significantly ($p<0.05$) reduced MCP-1 expression induced by pro-inflammatory cytokines. The inhibition was dose dependent. These data suggest that pro-inflammatory cytokines induce ROS production in beta cells through a NADPH oxidase mechanism that leads to beta cell dysfunction. Apocynin and DPI reduced beta cell intracellular ROS production that was induced by PICs (Supplement Figure 2).

Considering non-beta cell cellular models (Du, McLaughlin et al. 2002; Nardi, Feinmark et al. 2004; de Carvalho, Sadok et al. 2008; Sadok, Bourgarel-Rey et al. 2008; Wen, Gu et al. 2008), upstream activators of NADPH oxidase activity include 12-Lipoxygenase, an enzyme that has been implicated in the pathogenesis of diabetes development

(Bleich, Chen et al. 1999; McDuffie, Maybee et al. 2008) . Studies of a genetic deletion of 12-LO associate 12-LO activity with beta cell destruction and onset of diabetes. Further, 12-HETE, a lipid product of 12-LO activity directly induced beta cell dysfunction (Ma, Nunemaker et al. 2010). Induction of MCP-1 in peritoneal macrophages by 12-S-HETE was blocked by inhibitors of NADPH oxidase activity (apocynin or diphenyleneiodonium chloride) (Wen, Gu et al. 2008). These data indicate 12-LO activates NOX activity in a pathway leading to upregulation of MCP-1. However, this link has not previously been made in islet beta cells in relation to diabetes pathogenesis. To test this association, human donor islets were stimulated directly with 12-HETE, a bioactive lipid product of 12-LO activity. Treatment of human islets with 12-HETE (1nM) induced a significant 13-fold increase in NOX-1 expression ($p<0.05$) (Figure 3A). Further, a recently described selective inhibitor of 12-LO, cmpd #1 (IC50 12-LO 0.8μM) (Kenyon, Rai et al. 2011) was used to confirm the association between pro-inflammatory cytokines, 12-LO activity and NOX-1 expression. In the homogeneous beta cell line INS-1, the expression of NOX-1 induced by pro-inflammatory cytokines was significantly blocked by 5μM of the selective 12-LO inhibitor cmpd #1, and not by a structurally related molecule (Supplemental Figure 3) that is inactive (IC_{50} >70μM) in 12-LO inhibition (cpmd #E Figure 3B).

Translating the clinical relevance of these findings to human diabetic patients, islets from human type 2 diabetic donors were compared with non-diabetic donors. Islet dysfunction was confirmed both by increased gene expression of MCP-1 ($p<0.05$) and additionally by the presence of a defective first phase insulin release in a perfusion

glucose-stimulated-insulin-secretion test (data not shown). Unstimulated gene expression of NOX-1 from 6 representative non-diabetic donors (non DM) and five representative type 2 diabetes donors (T2DM) was determined. Plotted in Figure 4 are the normalized (to GAPDH) inverse Ct values for the two donor groups. A significantly higher expression of NOX-1 was detected in islets from type 2 diabetic donors ($p<0.05$). Further, supporting an interaction between 12-LO and NOX-1 expression in human diabetes, the protein expression for 12-LO was elevated in the T2DM group in Western blot analysis relative to the housekeeping protein, actin (data not shown). T2DM is associated with an increase in serum pro-inflammatory cytokines (Catalan, Gomez-Ambrosi et al. 2007; Steinberg 2007; Tilg and Moschen 2008; Al-Maskari, Al-Shukaili et al. 2010; Igoillo-Esteve, Marselli et al. 2010; Kang, Song et al. 2010; Su, Pei et al. 2010).

In summary, this study describes for the first time in islet beta cells a linked association in pro-inflammatory cytokine-induced beta cell dysfunction between 12-lipoxygenase, NADPH oxidase NOX-1 and reactive oxygen species. Inhibition of this pathway is a candidate target to preserve and protect beta cell mass in diabetes. Development of selective NOX-1 inhibitors could offer a new therapeutic strategy in diabetes.

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6. FIGURE LEGENDS

FIGURE 1: Pro-inflammatory cytokines induce expression of NOX-1 in islets and beta cell lines. Expression of NOX-1 gene was determined by qRT-PCR in unstimulated (untreated) cells and cells stimulated with a cocktail of TNFα, IL-1β and IFNγ (PIC treated). Data shows fold change in NOX-1 over control cells from three or greater experiments; * $p<0.05$.

FIGURE 2: NADPH Oxidase inhibitors, Apocynin and DPI block expression of MCP-1 induced by pro-inflammatory cytokines. Expression of MCP-1 in INS-1 cells was determined by qRT-PCR following stimulation with a cocktail of TNFα, IL-1β and IFNγ (PICs) with or without apocynin or DPI at the concentrations shown. Graph shows fold

change in MCP-1 expression relative to untreated cells. Data are from three experiments; *p<0.05, **p<0.01.

FIGURE 3: NOX-1 expression in islets and beta cells is induced by 12-Lipoxygenase. A), Nox-1 gene expression of human donor islets treated with 1nM 12-HETE. Graph shows fold change in qRT-PCR NOX-1 expression relative to untreated islets. B), Pro-inflammatory cytokine stimulation of INS-1 cells in the presence or absence of 5μM Cmpd#1 (a selective 12-LO-inhibitor) or 5μM CmpdE (a structurally related molecule that is not active in 12-LO inhibition). Graph shows percent of cytokine response. Data are from ≥3 experiments; * p<0.05, ** p<0.01.

FIGURE 4: NOX-1 expression is increased in islets from type 2 diabetic donors. Basal expression of NOX-1 was determined by qRT-PCR in unstimulated human donor islets, from non diabetic (non DM) or type 2 diabetic (T2DM). Graph shows inverse Ct value. Data are from six non diabetics and five type 2 diabetic donors; *p<0.05.

SUPPLEMENTAL FIGURE 1: Pro-inflammatory cytokines induce beta cell death and dysfunction. B), Human islets stimulated with a cocktail of TNFα, IL-1β and IFNγ (PIC) for 24 hours undergo apoptosis and necrosis (green, red and yellow fluorescence) unlike A), islets incubated in media alone for 24 hours. C), Induction of caspase-3 in mouse and human primary islets following treatment with PICs. Graph shows fold increase in qRT-PCR relative to untreated (control). D), Insulin secretion in human donor islets following 24 hours in media only (control) or media plus TNFα, IL-1β and

IFNγ (cytokines) in response to 3mM (Low) or 18mM (High) glucose. E), NOX-1 expression in INS-1 cells following four hour stimulation with IL-1β, TNFα, IFNγ alone or in combination (PICs). Graph shows fold increase in NOX-1 relative to untreated cells determined by qRT-PCR. Data are from ≥ 3 experiments; *p<0.05, ** p<001.

SUPPLEMENTAL FIGURE 2: ROS production induced pro-inflammatory cytokines is inhibited by apocynin and DPI. INS-1 cells were stimulated with a cocktail of TNFα, IL-1β and IFNγ (PIC) in the presence of 2mM apocynin (APO) or 10μM DPI. As a positive control, cells were also treated with 1mM H_2O_2. Graph shows fluorescence readout of DCF-DA expressed as a percentage of untreated cells (control). Data are from four experiments; **p<0.01.

SUPPLEMENTAL FIGURE 3: Chemical structures of compound 1 and compound E.