**Epoxyeicosatrienoic acids protect pancreatic beta cells against pro-inflammatory cytokine toxicity.**

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**Funding**

Daniel Grimes was supported with a studentship from the Faculty of Natural Sciences, Keele University. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Abstract**

Pro-inflammatory cytokines contribute to pancreatic beta cell death in the pathogenesis of type 1 diabetes mellitus (DM). Cytochrome P450-derived epoxyeicosatrienoic acids (EETs), produced by selective epoxidation of arachidonic acid, display anti-inflammatory activity in numerous disease models, in part through inhibition of NFκB activity. No studies have directly assessed their roles in cellular models of pancreatic beta cell death and therefore we aimed to investigate the cytoprotective effects of the EET isomers 8(9)-, 11(12)- and 14(15)-EET and their corresponding vicinal diols (dihydroxyeicosatrienoic acids, DHETs) in a model of pro-inflammatory cytokine-toxicity using the rat pancreatic beta cell line BRIN-BD11. Co-treatment of cells with a cocktail of pro-inflammatory cytokines (IL-1β, IFNγ and TNFα) caused a marked increase in caspase activation and a reduction in cell viability, effects attenuated by inclusion of each EET; this was also associated with a reduction in cytokine-induced NFκB activation and nitrite accumulation. Surprisingly, of the DHET derivatives of EETs, 8(9)-DHET conferred similar protective effects against cytokine-induced caspase activation. This data therefore highlights a novel role of EETs and a surprising activity of 8(9)-DHET in attenuating cytokine-toxicity in pancreatic beta cells.

**Keywords:** Epoxyeicosatrienoic acids, EETs, dihydroxyeicosatrienoic acids, cytokines, diabetes, beta cells

**Abbreviations:** Epoxyeicosatrienoic acid (EET), dihydroxyeicosatrienoic acid (DHET), cytochrome P450 (CYP450), soluble epoxide hydrolase (sEH), peroxisome-proliferator-activated-receptor-gamma (PPARγ), nuclear factor kappa B (NFκB).

**Introduction**

The pro-inflammatory cytokines interleukin-1 beta (IL-1β), interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα) contribute to pancreatic beta cell death and dysfunction in the pathogenesis of type 1 diabetes mellitus (DM). Exposure of pancreatic beta cells to these cytokines initiates an inflammatory intracellular signalling cascade, in part mediated by the downstream activation of nuclear factor kappa B (NFκB), mitogen-activated protein kinases (MAPK) and the JAK/STAT pathway [1]. Aberrant activation of these pathways is associated with the perturbation of multiple regulatory gene networks, including those responsible for increased nitrosative and oxidative stress, activation of an endoplasmic reticulum stress response and reduced beta cell function, culminating in beta cell apoptosis [1,2].

Growing evidence suggests an important anti-inflammatory action of epoxyeicosatrienoic acids (EETs) in various disease models, including hypertension, cardiac dysfunction, stroke, and hepatic dysfunction in models of inflammation and insulin resistance in DM and diet-induced ER stress [3,4,5]. EETs are produced by the action of cytochrome P450 (CYP450) epoxygenase 2J- and 2C- isoforms on arachidonic acid (AA), yielding four EET isomers; 5(6)-EET, 8(9)-EET, 11(12)-EET and 14(15)-EET, with two enantiomers of each [6]. The major route for further EET metabolism is their conversion to less active vicinal diols by soluble epoxide hydrolase (sEH), yielding the corresponding dihydroxyeicosatrienoic acids (DHETs), with sEH inhibitors being explored for their beneficial effects on vascular tone and cardioprotective activity in models of cardiovascular disease [7,8].

Mechanisms by which EETs exert their biological activity are not fully defined, though activation of peroxisome proliferator-activated receptor gamma (PPARγ) by EETs is well-established [7,9]. Alternatively, EETs are thought to signal in part through a putative extracellular G-protein coupled receptor (GPCR) and whilst several studies have supported a role for GPCR signalling in mediating EET action via Gαs-coupled signalling pathways [10,11,12], the molecular identification and characterisation of any such receptor remains elusive***.*** Nonetheless, anti-inflammatory actions of EETs as PPARγ agonists have been linked to reduced NFκB activity in several models, including vascular endothelial cells [9], with earlier studies observing that treatment with 11(12)-EET in the presence of TNF-α reduced nuclear accumulation of the p65 subunit of active NF-κB [13]. Pharmacological inhibition of sEH or its knock-down restored normoglycaemia and increased plasma insulin levels in streptozotocin (STZ)-induced diabetes in mice, associated with increased beta cell mass [14]. A later study observed similar effects, though was unable to directly attribute these effects to an increase in pancreatic or circulating EET:DHET ratios, despite demonstrating the presence of the AA epoxygenase, CYP2C11, in rat islets by immunoblotting [15]. Whilst recent studies have demonstrated that CYP2J2 overexpression restores glucose tolerance and improves inflammatory cytokine-induced hepatic insulin resistance in *db/db* mice [3], no studies have directly assessed a potential anti-inflammatory activity of EETs in pancreatic beta cells under conditions of pro-inflammatory cytokine administration. Thus the current study aimed to determine, for the first time, the cytoprotective and anti-apoptotic actions of EETs against pro-inflammatory cytokine toxicity in the rat pancreatic beta cell line BRIN-BD11.

**Methods**

*Cell culture*

BRIN-BD11 cells (a gift from Professor Noel Morgan, Exeter University) were cultured in RPMI-1640 media supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine, 100U/mL penicillin and 100µg/mL streptomycin and used between passage numbers 23-38. Cells were sub-cultured into 96-well plates (15,000 cells/well) or 24-well plates (40,000 cells/well) for subsequent experiments. All cytokine treatments were performed using 100U/mL IL-1β, 20U/mL IFNγ and 500U/mL TNFα (PeproTech EC).

*Preparation of EETs*

8(9)-EET, 11(12)-EET and 14(15)-EET and their corresponding DHETs (Cayman Chemicals) were supplied as a solution in ethanol (0.32mM). In some experiments, a methyl sulphonamide analogue of 14(15)-EET (14(15)-EET-SI) was used (Cayman Chemicals; supplied as a solution in methyl acetate), which lacks the free carboxylic acid group of the native EET and so is not susceptible to esterification to CoA and beta-oxidation. To concentrate all EETs and DHETs for later cell treatments, the solvent was evaporated under a stream of nitrogen and the free oil re-suspended in 100% ethanol (purged under nitrogen) to a concentration of 3.2mM. In congruence with methods used for the treatment of beta cell lines in the study of lipotoxicity and the cytoprotective activity of (poly)unsaturated fatty acids, EETs and DHETs were first prepared in FFA-free BSA at a Molar ratio of 3.3:1 by incubation at 37oC for 1 hour and further diluted into 1% FCS-containing RPMI-1640 with cytokines prior to cell treatment. The final concentration of all EETs and DHETs was 10µM and control cultures received appropriate concentrations of BSA and ethanol, with the final ethanol concentration not exceeding 0.5% (v/v).

*Vital dye staining*

Total and viable cell numbers post-treatment were assessed using Trypan blue vital dye exclusion. Briefly, detached and attached cells from cells treated in 24-well plates (the latter harvested by trypsin:EDTA digestion) were collected by centrifugation and the cell pellet concentrated into a volume of 200µL. The resulting cell suspension was diluted 1:2 with 0.4% (w/v) Trypan blue and the number of total and viable cells counted using a haemocytometer. Results were expressed as number of viable cells/mL (+/-SEM).

*Caspase activity*

Total caspase activity was assessed by flow cytometry using the MUSE™ MultiCaspase activity assay kit, according to manufacturer’s instructions (Merck). Results were expressed as the percentage of cells displaying total caspase activity (+/-SEM).

*NFκB luciferase assay*

NFκB activity was assessed using a NanoLuc® NFκB reporter vector (Promega) containing five copies of an NFκB response element upstream of NanoLuc® luciferase. Cells were seeded in black 96-well plates 24 hours before transfection with the NanoLuc® NFκB reporter vector (0.1ng/well) using Lipofectamine® 2000 (0.3µL/well); parallel transfections with the same ratio of vector DNA:Lipofectamine® using a GFP-expressing vector yielded ~60% transfection efficiency under these conditions. Following incubation with transfection complexes for 24 hours, cells were treated with pro-inflammatory cytokines alone, or in the presence of 10µM of each EET or DHET for a further 24 hours. Luminescence was measured using NanoGlo® reagent on a GloMax® luminometer, according to manufactures instructions (Promega), with results expressed as fold-change in relative luminescence units compared to vehicle-treated control.

*Nitrite determination*

To determine effects of EETs and DHETs on cytokine-induced nitrite accumulation, reflective of increased expression/activity of the inducible isoform of nitric oxide synthase (iNOS) and subsequent NO production, cells were seeded in 96-well plates and treated with cytokines alone, or in combination with EETs or DHETs. After 24 hours nitrite concentrations (µM) in cell supernatants were determined using Griess reagent (1% sulphanilamide in 5% orthophosphoric acid and 0.1% N-(1-Naphthyl)ethylenediamine dihydrochloride) relative to a sodium nitrite calibration graph (0-100µM), with absorbance measured at 540nm.

*Statistical analysis*

Graphed results are expressed as mean (+/- SEM). Statistical analysis was carried out using one-way or two-way ANOVA and Tukey’s or Bonferroni’s post hoc test with GraphPad Prism.

**Results**

Exposure of BRIN-BD11 cells to a cocktail of pro-inflammatory cytokines for 24 hours significantly reduced viable cell number (*P*<0.05), an effect attenuated by co-incubation with all EETs (Figure 1A), such that cell viability increased ~2-fold compared to cytokine treatment alone (*P*<0.05). Effects of EETs on cytokine-induced BRIN-BD11 cell death were mirrored in assays of caspase activation (Figure 1B-G), whereby cytokines increased the percentage of cells displaying caspase activation from 14% to 35.4%, reducing to control levels in the presence of each EET (*P*<0.05 vs. cytokine treatment alone). Given the widely reported view that DHET products of EET metabolism display considerably reduced biological activity to EETs, we sought to determine whether DHETs could confer any protection against cytokine toxicity in BRIN-BD11 cells. As would be expected, co-incubation with 11(12)-DHET and 14(15)-DHET failed to attenuate cytokine toxicity (*P*>0.05 vs. cytokine treatment alone), as assessed by both vital dye staining and caspase activation (Figure 2). However, surprisingly 8(9)-DHET did protect against cytokine toxicity, such that viable cell number was increased ~2-fold, an effect associated with a significant reduction in caspase activation from 41.3% to 21.7% (*P*<0.05), with activity comparable to 8(9)-EET.

To further explore potential mechanisms of EET and 8(9)-DHET action against pro-inflammatory cytokine toxicity, activation of the transcription factor NFκB was investigated using an NFκB luciferase reporter assay (Figure 3A). As expected, cytokine treatment increased NFκB activity ~4-fold compared to control and, in support of their action against cytokine-induced cell death and apoptosis, inclusion of all EETs significantly reduced NFκB activation by ~34% in response to cytokine treatment (Figure 3A, *P*<0.05). Similarly, NFκB activity was reduced in the presence of 8(9)- but not 11(12)- or 14(15)-DHET (Figure 3B). Increased nitrite levels (Figure 3C), reflective of cytokine-induced NO production, were also reduced in co-treatment with all EETs by ~62% compared to cytokine treatment alone (*P*<0.05), however, such a reduction was not observed in the presence of 8(9)-DHET (Figure 3D).

Further exploration of the mechanistic action of EETs and 8(9)-DHET was undertaken using pharmacological inhibition of PPARγ with the same experimental procedures (Figure 4A). However, the selective antagonist of PPARγ, T0070907, failed to reduce the ability of EETs or 8(9)-DHET to protect against cytokine-induced caspase activation. This suggests that EET actions, limiting cytokine-induced NFκB activation and subsequent induction of caspase activation and loss of cell viability, are independent of any agonistic action of EETs on PPARγ in this beta cell line model. In beta cell models of lipotoxicity relevant to type 2 DM, the free carboxylic group of mono and polyunsaturated fatty acids, required for their metabolic activation, has been shown to be dispensable for their ability to protect against palmitate-induced cell death [16]. Therefore, a methyl-sulphonamide (SI) derivative of 14(15)-EET, a metabolically stable derivative which lacks the free carboxylic acid group required for esterification to CoA (necessary for subsequent beta oxidation or membrane esterification), was compared with the free EET. Co-incubation of BRIN-BD11 cells with cytokines and 14(15)-EET, as expected, significantly attenuated the increase in caspase activation induced by cytokines (*P*<0.05), with similar effects observed in the presence of 14(15)-EET-SI (*P*<0.05 vs cytokines alone), suggesting that the free carboxylic acid group and subsequent esterification to CoA is not required for mechanisms of EET action in this model (Figure 4B).

**Discussion**

Anti-inflammatory activities of EETs are widely reported in a number of disease models however, no studies have directly assessed their role in cellular models of beta cell cytotoxicity. We report here the novel observation that EETs can protect against pro-inflammatory cytokine toxicity in the rat pancreatic beta cell line, BRIN-BD11. In support of observations in other models, these effects were related to a reduction in cytokine-induced NFκB activation [3,9,13], decreased NO production and nitrite accumulation. Whilst several mechanisms for EET action have been reported, a well-established role is their activity as PPARγ agonists [3,7,9] however, in the current model, pharmacological inhibition of PPARγ failed to block the protective effects of EETs against cytokine toxicity. This may indicate PPARγ-independent actions of EETs in BRIN-BD11 cells and similarly others have observed that the anti-inflammatory actions of EETs in terms of iNOS activity in smooth muscle cells appears to be independent to the activation of all thee PPAR isoforms (Thompson and Bishop-Bailey, unpublished observations, cited in [17]).

Although it is widely reported that DHET products of sEH action on EETs are considerably less active, fewer studies have directly assessed the biological role of 8(9)-EET/DHET in cellular models, with most reporting on the action of 11(12)- and 14(15)-EETs [18]. Similarly, studies on the endogenous production of EETs through over-expression of relevant CYP450- epoxygenases have largely attributed observed effects to production of 11(12)- and 14(15)-EETs. Therefore we also report here on the surprising observation that the more metabolically stable derivative of 8(9)-EET, 8(9)-DHET, displays comparable activity to EETs in the extent of apoptosis suppression in response to inflammatory cytokine exposure. Whilst CYP450 enzymes are capable of producing all four EET isomers, the 5(6)-EET species is known to be highly reactive and unstable due to the proximity of the epoxide group to the carboxylic acid group, facilitating rapid hydration to the DHET, hence a comparison between any effects of 5(6)-EET and 5(6)-DHET was not included in the current study. Studies measuring the relative ratios of EETs produced by CYP450 enzymes have reported that 14(15)-, 11(12)- and 8(9)-EETs are produced in a ratio of 2.3:1.0:0.5, respectively [6], thus the 8(9)-EET species (and therefore any subsequent conversion to 8(9)-DHET) may represent a minor fraction of the endogenous EET/DHET pool. That 8(9)-DHET could rescue BRIN-BD11 cells from cytokine toxicity warrants further investigation, since sEH inhibitors have been shown to partially enhance the mitogenic activity of 8(9)-EET in endothelial cells [18] and sEH inhibitors enhanced beta cell mass in STZ-induced DM [14,15]; though these latter studies did not observe an increase in the relative ratios of EETs:DHETs, nor consider further potential mechanisms of EET action. Some have reported biological activity of 11(12)- and 14(15)-DHET in Ca2+-activated K+ channels in isolated rat coronary arterial myocytes [19] and the hypoxic response of human hepatoma cells and umbilical artery endothelial cells [20], and whilst others have shown that 14(15)-DHET has weak binding affinity to a putative EET membrane receptor [11], no reports have assessed 8(9)-DHET in this context.

Regardless of PPARγ activity, all EETs and 8(9)-DHET conferred significant protection against cytokine toxicity in the current model, and whilst EET-mediated inhibition of NFκB activity has been attributed, in part, to PPARγ activation [9], EETs are also suggested to signal through a putative EET receptor, though the molecular identity of this, and whether this represents an extra- or intracellular receptor, remains unclear. However, a radiolabelled 14(15)-EET analogue has been shown to bind at the plasma membrane in U937 cells [11], effects associated with increased cytosolic cAMP, suggesting that EETs may signal in part through an, as yet unidentified, GPCR(s). Furthermore, a recent study screening EET action at 105 known GPCRs failed to identify a single high-affinity receptor, instead demonstrating activity at a number of low-affinity prostaglandin (PG) receptors (including PTGER2, PTGER4, PTGFR, PTGDR and PTGER3IV), concluding that EETs may signal through multiple GPCRs [10]. The functional significance of prostaglandins in beta cell models is widely reported [21] and whilst the role of PGE2 in insulin secretion (which signals through PTGER2, PTGER4 and PTGER3IV) remains unclear, activation of these receptors (and PTGDR by PGD) leads to increased cAMP, supporting a possible role in EET-Gαs signalling [10,11]. It is therefore conceivable that, in the current model, EET action is similarly mediated partly through activation of multiple GPCRs, effects associated with an inhibitory action on NFκB activation independent to PPARγ activity. In support of this hypothesis, cAMP levels have also been linked to an inhibitory effect on NFκB activity (mediated via protein kinase A activation) [22] and the GPCR kinase 5 has been shown to inhibit NFκB activation in endothelial cells, attributed to nuclear retention of IκBα and impaired transcriptional activity of NFκB [23]. This hypothesis is also consistent with studies highlighting important autocrine and paracrine actions of EETs [24] and with our observation that the metabolically stable EET analogue 14(15)-EET-SI, which lacks the free carboxylic acid group for esterification to CoA, conferred similar protection against cytokine-induced apoptosis in BRIN-BD11 cells to 14(15)-EET.

Collectively, this study reports the novel observation that EETs and 8(9)-DHET display anti-inflammatory activity in a beta cell model and can protect clonal pancreatic beta cells from pro-inflammatory cytokine-induced apoptosis and cell death, mediated in part by a reduction in NFκB activity and nitrite accumulation. Given the uncertainty in current literature on the mechanisms of EET/DHET action, further studies to delineate such mechanisms, including in beta cell models, are warranted. Whilst the molecular identity of any extracellular EET receptor(s) is yet to be determined, whether such receptors display differential affinity towards distinct EET and DHET isomers could account for the novel observation in this study that 8(9)-DHET conferred similar protection against cytokine-induced cell death as 8(9)-EET, unlike other DHET isomers. As a more metabolically stable product of EET metabolism, the 8(9)-DHET species should provide a starting point for further exploration of such modes of action in a beta cell context. Whether these results can be recapitulated in islet cultures and preserve islet function in *in vivo* models of type 1 and 2 DM (in a pathophysiological context) will shed further light on these hitherto largely unexplored (in a beta cell context) anti-inflammatory eicosanoids. A better understanding of mechanisms of EET/DHET action in beta cells may also provide novel therapeutic targets to preserve functional beta cell mass in DM, as well as in the attenuation of intra-islet inflammatory responses and mitigation of islet loss in the immediate post-transplant period in current models of islet transplantation [25]. Our on-going work is also exploring the role of EETs in cellular models of lipotoxicity relevant to the pathogenesis of type 2 DM and the role of CYP450 enzymes in the endogenous production of EETs.

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**Figure 1. EETs protect BRIN-BD11 cells against cytokine toxicity.** Cells were treated with cytokines alone, or in co-incubation with 10µM of either 8(9)-, 11(12)-, or 14(15)-EET for 24 hours before cell viability was determined using Trypan blue vital dye exclusion (A), or caspase activation assessed by flow cytometry using the MUSE™ MultiCaspase assay (B-G). Results are expressed as mean +/- SEM (A-B), where \*P<0.05 vs. control and #P<0.05 vs. cytokine treatment alone, as determined by one-way ANOVA and Tukey’s post-test, n=3. Representative plots from the flow cytometry data for caspase activity are shown in panels C-G.

**Figure 2. 8(9)-DHET but not 11(12)- or 14(15)-DHET protects BRIN-BD11 cells against cytokine toxicity.** Cells were treated with cytokines alone, or in co-incubation with 10µM of either 8(9)-, 11(12)-, or 14(15)-DHET for 24 hours before cell viability was determined using Trypan blue vital dye exclusion (A), or caspase activation assessed by flow cytometry using the MUSE™ MultiCaspase assay (B-G). Results are expressed as mean +/- SEM (A-B), where \*P<0.05 vs. control and #P<0.05 vs. cytokine treatment alone, as determined by one-way ANOVA and Tukey’s post-test, n=3. Representative plots from the flow cytometry data for caspase activity are shown in panels C-G.

**Figure 3. EETs and 8(9)-DHET reduce cytokine-induced NFκB activation in BRIN-BD11 cells.** A-B: Cells were transfected with a NanoLuc® NFκB luciferase reporter vector 24 hours prior to treatment with cytokines alone, or in co-incubation with EETs or DHETs, for a further 24 hours, with luciferase activity determined using NanoGlo® luciferase substrate and results expressed as fold change (+/-SEM) compared to control. C-D: nitrite levels in culture supernatants (µM) were determined using the Griess assay following treatment of cells with cytokines alone (■), or in co-incubation (□) with EETs or DHETs (nitrite levels for untreated controls were ~1.3µM. \*P<0.05 vs. control and #P<0.05 vs. cytokines alone, as determined by one-way ANOVA and Tukey’s post-test (A-B n=5) or two-way ANOVA and Bonferroni’s post-test (C-D n=3).

**Figure 4. EET and 8(9)-DHET effects are independent to PPARγ activation or their esterification to CoA.** Cells were treated with cytokines alone, or in co-incubation with 10µM of either 8(9)-, 11(12)-, or 14(15)-EET in the presence (□) or absence (■) of 2µM of the PPARγ antagonist T0070907 (A) or in the presence of 14(15)-EET-SI (B) for 24 hours before caspase activation was assessed by flow cytometry using MUSE™ MultiCaspase assay. Results are expressed as mean +/- SEM, where \*P<0.05 vs. control and #P<0.05 vs. cytokines alone, as determined by two-way ANOVA and Bonferroni’s post-test (A) or one-way ANOVA and Tukey’s post-test (B), n=3.

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