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Article

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Abstract

Aims/hypothesis We have previously shown that oxidative stress plays a causal role in beta cell dysfunction induced by fat. Here, we address whether the proinflammatory kinase Inhibitor of (nuclear factor) κB kinase β (IKKβ), which is activated by oxidative stress, is also implicated.

Methods Fat (oleate or olive oil) was infused intravenously in Wistar rats for 48 h with or without the IKKβ inhibitor salicylate. Thereafter, beta cell function was evaluated in vivo using hyperglycaemic clamps or ex vivo in islets isolated from fat-treated rats. We also exposed rat islets to oleate in culture, with or without salicylate and 4(2′-aminoethyl)amino-1,8-dimethylimidazo(1,2-a)quinoxaline; BMS-345541 (BMS, another inhibitor of IKKβ) and evaluated beta cell function in vitro. Furthermore, oleate was infused in mice treated with BMS and in beta cell-specific *Ikkb*-null mice.

Results 48h infusion of fat impaired beta-cell function *in vivo*, assessed using the disposition index (DI; μmol kg^{-1} min^{-1} glucose divided by pmol insulin multiplied by nmol C-peptide), in rats (Saline: 1.41 ± 0.13; Oleate: 0.95 ± 0.11; Olive oil [OLO]: 0.87 ± 0.15; $p<0.01$ both fats vs. saline) and in mice (Saline: 2.51 ± 0.39; Oleate: 1.20 ± 0.19; $p<0.01$ vs. saline) and *ex vivo* (units are pmol insulin $islet^{-1}$ h^{-1}) in rat islets (Saline: 1.51 ± 0.13; Oleate: 1.03 ± 0.10; OLO: 0.91 ± 0.13; $p<0.001$ both fats vs. saline) and the dysfunction was prevented by coinfusion of salicylate in rats (Oleate + Salicylate: 1.30 ± 0.09; OLO + Salicylate: 1.33 ± 0.23) or BMS in mice (Oleate + BMS: 2.25 ± 0.42) *in vivo* and by salicylate in rat islets *ex vivo* (Oleate + Salicylate: 1.74 ± 0.31; OLO + Salicylate: 1.54 ± 0.29). In cultured islets, 48h exposure to oleate impaired beta-cell function (Control: 0.66 ± 0.12; Oleate: 0.23 ± 0.03; $p<0.01$ vs. saline), an effect prevented by both inhibitors (Oleate + Salicylate: 0.98 ± 0.08; Oleate + BMS: 0.50 ± 0.02). Genetic inhibition of IKKβ also prevented fat-induced beta-cell dysfunction *ex vivo* (Control saline: 0.16 ± 0.02; Control oleate:

0.10 ± 0.02; Knockout oleate: 0.17 ± 0.04; $p<0.05$ control saline vs. control oleate) and *in vivo* (Control saline: 3.86 ± 0.40; Control oleate: 1.95 ± 0.29; Knockout oleate: 2.96 ± 0.24; $p<0.01$ control saline vs. control oleate).

Conclusions/interpretation Our results demonstrate a causal role for IKKβ in fat-induced beta cell dysfunction in vitro, ex vivo and in vivo.

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Keywords Beta cell dysfunction, IKKβ, Inflammation, In vivo, Lipotoxicity, Oleate, Olive oil, Oxidative stress

Abbreviations

AMPK	5′ Adenosine monophosphate-activated protein kinase
BMS	4(2′-Aminoethyl)amino-1,8-dimethylimidazo(1,2-a)quinoxaline; BMS-345541
COX	Cyclooxygenase
Cre	Cre recombinase
DI	Disposition index
GINF	Glucose infusion rate
IκBα	Inhibitor of (nuclear factor) κB α
IKKβ	Inhibitor of (nuclear factor) κB kinase β
$IKK\beta^{\Delta beta\ cell}$	Beta cell-specific IKK beta-deficient
IL-1RA	IL-1 receptor antagonist
INOS	Inducible nitric oxide synthase
MCP-1	Monocyte chemoattractant protein 1
M/I	Insulin sensitivity index (glucose metabolism [*M*]/plasma insulin [I])
OLO	Olive oil
PGE2	Prostaglandin E2
RIP2	Rat insulin 2 promoter
ROS	Reactive oxygen species

Introduction

NEFA have been shown to both stimulate and impair insulin secretion depending on the duration of beta cell exposure [1]. Prolonged (>24 h) exposure is inhibitory and involves oxidative stress, endoplasmic reticulum stress and inflammation [1].

Lipotoxic effects on the beta cell, i.e. the decrease in beta cell function and mass induced by chronically elevated NEFA, play a role in the pathogenesis of type 2 diabetes, at least in predisposed individuals [1]. Previously, we demonstrated that oxidative stress mediates fat-induced beta cell dysfunction in vivo in rats [2] and in humans [3]. Oxidative stress activates Inhibitor of (nuclear factor) κB kinase β (IKKβ), which, by phosphorylating the Inhibitor of (nuclear factor) κB α (IκBα), activates the transcription factor NFκB. The effect of IKKβ/NFκB on beta cell function is controversial. NFκB is important for cell survival and there are reports that NFκB is beneficial for glucose-stimulated insulin secretion [4], unless activated by cytokines [5]. However, IKKβ which, in addition to activating NFκB, induces serine phosphorylation of IRSs [6], decreases beta cell function [7]. It is also controversial whether fat activates IKKβ/NFκB in beta cells: although fatty acids did not activate NFκB in INS-1 or primary rat beta cells in one study [8], lipotoxicity was associated with NFκB activation, and palmitate-induced apoptosis was inhibited by an IKKβ inhibitor in INS-1 beta cells in another study [9]. The in vivo effect of fat on beta cell IKKβ/NFκB has not been investigated previously.

To address the role of IKKβ in fat-induced beta cell dysfunction in vivo, rats were infused i.v. with fat to elevate plasma NEFA 50–100% (elevation seen in obesity [10]) with or without the IKKβ inhibitor [11] salicylate for 48 h. Monounsaturated fat (oleate or olive oil) was infused, as in our previous study showing a role of oxidative stress in beta cell dysfunction [2]. Although oleate has been found to protect beta cells against palmitate-induced toxicity in in vitro studies

[12], the prolonged effect of oleate by itself on beta cell function is mainly inhibitory [2,13]. After 48 h infusion, beta cell function was evaluated in vivo using hyperglycaemic clamps, or ex vivo in isolated islets. We also used the specific IKKβ inhibitor 4(2′-aminoethyl)amino-1,8-dimethylimidazo(1,2-a)quinoxaline; BMS-345541 (BMS) [14] in hyperglycaemic clamps performed after 48 h oleate infusion in mice. In addition, we exposed rat islets for 48 h to oleate with or without salicylate or BMS in vitro. Last, we performed oleate infusion in beta cell-specific IKKβ-deficient ($IKK\beta^{\Delta beta\ cell}$) mice, followed by evaluation of beta cell function in vivo using hyperglycaemic clamps or ex vivo in isolated islets. In all these models, IKKβ inhibition prevented fat-induced beta cell dysfunction.

Methods

Animals All procedures were approved by the Animal Care Committee of the University of Toronto and conducted according to the Canadian Council on Animal Care Guidelines and the appropriate sample sizes were determined prior to the studies (see also Electronic supplementary material [ESM]). Female Wistar rats (250-300g, corresponding to 9-11 weeks of age, Charles River, Senneville, QC, Canada) were used, as in our previous studies [2,15]. Female C57BL/6 mice (22-25g, corresponding to 14-16 weeks of age, Jackson Laboratory, Bar Harbor, ME, USA) and male RIP2 (rat insulin 2 promoter)-Cre recombinase (Cre) positive or negative *Ikkb* floxed mice were also used [16]. Female wild-type C57BL/6 mice underwent the BMS infusion studies. For studies in beta cell-specific *Ikkb*-null mice, $Ikkb^{F/F}$ mice on a C57BL/6 background were crossed with RIP2-Cre mice (also on a C57BL/6 background) obtained from Jackson Laboratory to generate RIP2-Cre positive or RIP2-negative $Ikkb^{F/+}$ mice, which were interbred to generate RIP2-Cre^{-}:$Ikkb^{F/F}$ (floxed controls) and RIP2-Cre^{+}:$Ikkb^{F/F}$ ($Ikkb^{\Delta beta\ cell}$) mice. These mice were used for experiments at 11-13 weeks of age. Cre-mediated recombination was confirmed by PCR [17].

Surgery and i.v. infusion Surgery was performed under general anaesthesia (isoflurane, to effect). Carotid artery and jugular vein cannulation of rats has been described previously [2,15]. After 3 days' post-surgical recovery, the rats were randomised to the following 48 h infusions: (1) NaCl (154 mmol/l; 5 μl/min) as control; (2) fat, either oleate (1.3 μmol/min) prepared as in our previous studies [2,15] or 20% (vol./vol.) olive oil (OLO; 5.5 μl/min) prepared as in Dobbins et al [18]; (3) oleate or OLO + salicylate (0.7 μmol kg^{-1} min^{-1}, the dose that reversed insulin resistance in Park et al [19]); or (4) salicylate only. Olive oil is a triacylglycerol mixture containing 75% oleate, and 16% saturated fat. Heparin was added to olive oil to a final concentration of 50 U/ml to activate

lipoprotein lipase, which releases NEFA and glycerol from the triacylglycerol mixture of olive oil. We have shown that BSA, the vehicle for oleate, has no effect on insulin secretion [15,20]; heparin [21] and glycerol [22] also have no effect. A two-step hyperglycaemic clamp or islet isolation was performed after the infusion period.

Mouse jugular vein cannulation is described in Koulajian et al [20]. In mice, oleate (0.4 μmol/min)/equivolume saline was infused for 48 h [20], starting 4-5 days after surgery with or without BMS (0.12 mmol kg^{-1} day^{-1}) [14]. After 48 h infusion, mice received hyperglycaemic clamps. Sampling was through the tail vein.

Hyperglycaemic clamp Insulin secretion was determined by measuring plasma insulin and C-peptide during a two-step (13 and 22 mmol/l) hyperglycaemic clamp in rats [2] and a one-step (22 mmol/l) hyperglycaemic clamp in mice [20]. Insulin sensitivity (*M*/I, where *M* is glucose metabolism and I is plasma insulin) and beta cell function (disposition index [DI]) were assessed as described in the ESM.

Hyperinsulinaemic–euglycaemic clamp Insulin sensitivity was determined using the gold-standard technique, i.e. hyperinsulinaemic–euglycaemic clamp [19].

Ex vivo studies Islets of the in vivo infused rats and *Ikkb*$^{\Delta\text{beta cell}}$ mice were isolated and insulin secretion studies were performed as described previously [2]. ELISA for phosphorylated IκBα and active nuclear NFκB (Active Motif, Carlsbad, CA, USA), reactive oxygen species (ROS) measurements (see ESM) and RT-PCR were also performed in rat islets. Total rat islet RNA was prepared as described previously [23,24] according to the manufacturer's instructions (Qiagen, Hombrechtikon, Switzerland), and was reverse transcribed using random hexamers. Mouse primers for *Il1b*, *Tnfa* (also known as *Tnf*), monocyte chemoattractant protein 1 (*Mcp1*; also known

as *Ccl2*), IL-1 receptor antagonist (*Il1ra;* also known as *Il1rn*), *Tgfb* (also known as *Tgfb1*), the macrophage marker *Cd68*, and cyclooxygenase (*Cox*)*2* (also known as *Ptgs2*) (see ESM Table 1) from Applied Biosystems (Foster City, CA, USA) were used and quantitative PCR was done with a fluorescein amidite (FAM)-based reference dye using commercial TaqMan gene expression assays and the 7500 Fast Real-Time PCR System according to the manufacturer's protocol (Applied Biosystems). Changes in mRNA expression were calculated using difference in C_t values, and normalised to the housekeeping gene 18S [23,24].

Studies in cultured islets Rat islets were cultured for 48 h in RPMI 1640 without antioxidants, containing 0.4 mmol/l oleate in 0.5% NEFA-free BSA with or without 0.25 mmol/l salicylate [7] or 3 μmol/l BMS, a dose based on pilot dose–response studies. Islets were also cultured in control/oleate media with or without the COX-2 inhibitor SC-236 (10 μmol/l, based on Castaño et al [25]) or the COX-1 inhibitor SC-560 (100 μmol/l, based on Smith et al [26]). Thereafter, glucose-stimulated insulin secretion was assessed. Prostaglandin E2 (PGE2) in the medium was measured via ELISA (Enzo Life Sciences, Farmingdale, NY, USA).

Western blots Western blots were performed as previously described [19] using antibody against phospho-Ser307-IRS-1 (Millipore, Billerica, MA, USA, 07-247, raised in rabbit, RRID:AB_310463, 1:500), total IKKβ (Cell Signaling Technology, Danvers, MA, USA, 2370, raised in rabbit, RRID:AB_2122154, 1:100), phospho-Thr172-5′ adenosine monophosphate-activated protein kinase (AMPK)α (Cell Signaling Technology, 2535, raised in rabbit, RRID:AB_331250, 1:1000), total AMPKα (Cell Signaling Technology, 2793, raised in mouse, RRID:AB_915794, 1:1000), total IκBα (Santa Cruz Biotechnology, Dallas, TX, USA, sc-371, raised in rabbit, RRID:AB_2235952, 1:250), α-actinin (Cell Signaling Technology, 3134S, raised

in rabbit, RRID:AB_2223798, 1:1000), β-actin (Abcam, Cambridge, UK, ab6276, RRID:AB_2223210, raised in mouse, 1:10,000) or γ-tubulin (Sigma-Aldrich, St. Louis, MO, USA, T6557, RRID:AB_477584, raised in mouse, 1:1,000) (see also ESM). The antibodies had been validated in previous studies carried out in the laboratory (see ESM).

Plasma assays Plasma glucose in rats was measured on a Beckman Analyser II (Beckman, Fullerton, CA, USA) and in mice was measured on a HemoCue Glucose 201 Analyser (HemoCue, Brea, CA, USA). Plasma NEFA were measured with an enzymatic colorimetric kit (Wako Industries, Neuss, Germany). Radioimmunoassays specific for rat/mouse insulin and C-peptide (Linco, St Charles, MO, USA) were used to determine their plasma concentrations. Plasma triacylglycerol levels were measured by a colorimetric kit (Boehringer Mannheim, Laval, QC, Canada).

Statistics Data are means ± SEM. One-way non-parametric ANOVA for repeated measurements followed by Tukey's test was used to compare treatments. Calculations were performed using SAS version 8.0 (Cary, NC, USA).

Results

Hyperglycaemic clamp in rats Rats were infused i.v. with saline, oleate or OLO, with or without the IKKβ inhibitor salicylate. After 48 h infusion, levels of plasma NEFA were ~1.5-fold higher with oleate or OLO and triacylglycerol levels were elevated by OLO (Table 1). Oleate, OLO or salicylate did not affect plasma glucose or insulin (Table 1). Following the 48 h infusions, we evaluated beta cell function in vivo using two-step hyperglycaemic clamps (13 mmol/l and 22 mmol/l, Fig. 1a,b). The glucose infusion rate (GINF) necessary to maintain the clamp was lower with oleate or OLO, suggesting reduced insulin secretion and/or sensitivity (Fig. 1c,d). With oleate + salicylate or OLO + salicylate, GINF was similar to control (Fig. 1c,d).

Clamp plasma insulin was lower with oleate infusion and was restored with oleate + salicylate, whereas insulin was not different from control with OLO and OLO + salicylate (Fig. 1e,f). Clamp C-peptide showed the same pattern as insulin (Fig. 1g,h), indicating unchanged absolute insulin secretion in OLO-treated groups.

The insulin sensitivity index (*M*/I = GINF/insulin [27]) was not affected by oleate (Fig. 2a), as previously found in the same model [2,15], and was also unaffected by salicylate. With OLO, *M*/I was reduced, indicating insulin resistance (Fig. 2b). The decrease in *M*/I was prevented by salicylate. The evaluation of beta cell function in vivo should take into account the ambient insulin sensitivity, as normal beta cells increase insulin secretion in response to insulin resistance along a hyperbola, characterised by a constant DI [28]. The DI is an established index of beta cell function [28] that we have previously validated in rodents [20,24]. The DI was impaired in both oleate and OLO groups (Fig. 2c,d), indicating reduced beta cell function; the impairment was completely prevented by salicylate. Salicylate alone had no effect on DI.

Hyperinsulinaemic–euglycaemic clamp When evaluated by hyperinsulinaemic–euglycaemic clamp, insulin sensitivity tended to be decreased by oleate (n=4), and was significantly decreased by OLO (n=3) compared with saline (n=4) (ESM Fig. 1).

Ex vivo studies in rat islets Glucose-stimulated insulin secretion from islets isolated from rats i.v. infused with oleate or OLO was markedly impaired, but secretion was restored with salicylate (Fig. 3a,b). Oleate or OLO increased phosphorylated IκBα (Fig. 3c,d) and active nuclear NFκB (Fig. 3e,f), effects that were not apparent in the presence of salicylate.

Oleate increased the levels of islet mRNA for *Il1b, Tnfa, Mcp1, Il1ra, Tgfb, Cd68* and *Cox2*; the effect of oleate was prevented by salicylate (Table 2). Interestingly, salicylate alone decreased these markers. The signal for *Inos* (inducible nitric oxide synthase, also known as *Nos2*) mRNA was undetectable in all groups. Oleate (n=6) increased islet ROS compared with saline (n=5); however, ROS levels were not reduced by adding salicylate to oleate (n=8), and salicylate alone had no effect (n=4) (ESM Fig. 2).

Hyperglycaemic clamp in mice We used 48 h oleate infusion in mice, with or without BMS, which is a much more potent and specific IKKβ inhibitor than salicylate, at a dose previously found to inhibit IKKβ in vivo [14]. After the 48 h infusion and prior to the hyperglycaemic clamp the groups treated with oleate had higher plasma NEFA (oleate 1.205 ± 0.156 mmol/l; oleate + BMS 1.177 ± 0.136 mmol/l) than the groups infused with saline (0.761 ± 0.206 mmol/l) or BMS alone (0.689 ± 0.070 mmol/l). The glucose level was raised to 22 mmol/l in all groups (Fig. 4a). The GINF necessary to maintain the clamp was lower in oleate-infused animals but was similar to saline with oleate + BMS (Fig. 4b). Clamp insulin and C-peptide were not lower in oleate-treated mice than in control mice (Fig. 4c,d), in contrast to our oleate model in rats, but similar to our previous studies in mice [20] and our olive oil model in rats. Accordingly, the sensitivity index

M/I [27] was lower in oleate-treated mice (Fig. 4e). Basal and clamp insulin and C-peptide levels were higher in the groups treated with BMS. BMS had no significant effect on *M*/I when added to oleate but on its own decreased *M*/I. DI was decreased with oleate infusion, whereas BMS completely prevented the oleate-induced decrease. BMS alone had no effect on DI (Fig. 4f).

Studies in beta cell-specific IKKβ-deficient mice We also used *Ikkb*$^{\Delta\text{beta cell}}$ and littermate floxed control mice to determine whether genetic silencing of IKKβ protects from fat-induced beta cell dysfunction ex vivo and in vivo. *Ikkb* deletion in islets of *Ikkb*$^{\Delta\text{beta cell}}$ mice was confirmed by immunoblotting (Fig. 5a) and there was a suggestion of partial deletion in the hypothalamus (Fig. 5b). There was no significant difference in weight between ~13 week old control (29.7±0.7g, n=14) and *Ikkb*$^{\Delta\text{beta cell}}$ (28.5±0.8 g, n=13) mice. Oleate infusion for 48 h elevated plasma NEFA approximately threefold (Fig. 5c). Glucose-stimulated insulin secretion from islets isolated from oleate-infused controls was impaired and this impairment was prevented in *Ikkb*$^{\Delta\text{beta cell}}$ mice (Fig. 5d).

Before hyperglycaemic clamping, NEFA were elevated approximately twofold in the oleate-infused groups (Fig. 6a). During hyperglycaemic clamps, the decreased *M*/I found in oleate-infused controls was not observed in *Ikkb*$^{\Delta\text{beta cell}}$ mice (Fig. 6b), which may be explained by partial deletion of hypothalamic *Ikkb* driven by RIP2. Importantly, *Ikkb*$^{\Delta\text{beta cell}}$ mice were protected from the decrease in DI induced by oleate in controls (Fig. 6c).

In vitro studies in islets To completely rule out that the effect of salicylate or BMS on beta cell dysfunction was mediated by systemic effects, we performed in vitro studies. Rat islets were cultured for 48 h in control or oleate-containing medium, with or without salicylate. Oleate decreased glucose-stimulated insulin secretion, and salicylate prevented this decrease (Fig. 7a).

Oleate decreased total IκBα (a marker of IKKβ activity as phosphorylated IκBα is degraded) and the decrease was prevented with salicylate (Fig. 7b). Salicylate is known to activate AMPK by preventing its dephosphorylation [29] and AMPK activation can result in IKKβ inhibition [30], though salicylate has also been reported to directly inhibit IKKβ [11]. There was a tendency for oleate to decrease phosphorylation of AMPK, which appeared to be prevented with salicylate, but there was no significant effect (Fig. 7c). BMS, which inhibits IKKβ in AMPKα-null cells [31], prevented oleate-induced beta cell dysfunction similar to salicylate (Fig. 7d).

The IKKβ/NFκB pathway can mediate oleate-induced beta cell dysfunction by at least three mechanisms: (1) impairment of beta cell insulin signalling via serine phosphorylation of IRS [6]; (2) increase in COX-2-derived PGE2 [32]; and (3) production of NO through induction of INOS [33]. However, *Inos* (also known as *Nos2*) mRNA was undetectable in our islets in the ex vivo studies. Oleate increased ser307-phosphorylated IRS-1 in cultured islets, an effect prevented by salicylate (Fig. 8a). Oleate also increased PGE2 release in media and this was prevented by salicylate (Fig. 8b). We also treated islets with the COX-2 inhibitor SC-236, which prevented the secretory defect induced by oleate (Fig. 8c), whereas the COX-1 inhibitor SC-560 did not (insulin secretion values at 22 mmol/l glucose relative to control: control = 1.00±0.33; oleate = 0.59±0.17; oleate + SC-560 = 0.14±0.06; SC-560 = 0.51±0.1; n=2-4/group).

Discussion

We examined the effects of prolonged NEFA exposure with or without IKKβ inhibitors on beta cell function in vivo, ex vivo and in vitro. We used our in vivo models of lipotoxicity in rats [2,15] and mice [20]. These are models of beta cell dysfunction, as beta cell mass is not decreased by 48 h fat infusion [20,24] and apoptosis is not increased [34]. During hyperglycaemic clamps in vivo, both insulin and C-peptide levels were lower in rats treated with oleate, indicating reduced insulin secretion; reduced glucose-stimulated insulin secretion was also found in isolated islets ex vivo. With olive oil, glucose-stimulated insulin secretion ex vivo was reduced but insulin and C-peptide levels (indices of absolute insulin secretion) during hyperglycaemic clamps were unaffected. The different absolute insulin secretion between oleate and olive oil is explained by the effect of olive oil to induce a greater degree of insulin resistance. This was demonstrated using hyperinsulinaemic–euglycaemic clamps, which are the gold-standard assessment for insulin sensitivity in vivo. In vivo, the beta cell compensates for insulin resistance by increasing secretion. In the absence of insulin resistance, absolute insulin secretion corresponds to DI. However, in the presence of insulin resistance, DI rather than absolute insulin secretion should be taken as a measure of beta cell function, which includes the ability of the beta cell to compensate for insulin resistance. DI was impaired by both oleate and olive oil, showing a decrease in beta cell function with both types of fat, consistent with the ex vivo results with islets. The different effects of oleate and olive oil on insulin sensitivity may reflect the amount of saturated fat in olive oil (16%) and/or the plasma triacylglycerol elevation induced by olive oil [35].

Our results showing that fat-induced beta cell dysfunction was prevented by salicylate in vivo and ex vivo in isolated islets suggest a role for inflammatory pathways involving IKKβ in lipid-induced beta cell dysfunction.

Inflammatory pathways are known to be activated in beta cell glucotoxicity [36] and may enhance lipotoxicity [37]. Our previous results show that oxidative stress plays a causal role in beta cell dysfunction induced by monounsaturated fat [2]. ROS are known activators of IKKβ which, in addition to phosphorylating IκBα and thereby activating NFκB, phosphorylates IRS, thus inhibiting insulin signalling. In our ex vivo study, salicylate prevented the increase in phosphorylated IκBα and nuclear active NFκB induced by oleate or olive oil, although, interestingly, salicylate alone had no effect at this dose, as previously seen in the liver [19]. An increase in ROS was induced by oleate, but salicylate did not prevent this effect. This suggests IKKβ is a downstream effector required for the previously demonstrated effect of oxidative stress to induce beta cell dysfunction [2]. IKKβ activation could also be unrelated to oxidative stress in the case of olive oil, which contains saturated fat. Saturated fatty acids can activate IKKβ via toll-like receptors (TLR) 2 and 4. *Tlr4*-null mice are protected from beta cell dysfunction induced by palmitate [38] and *Tlr2*-null mice are protected from beta cell dysfunction induced by high-fat diet [23]. Although saturated fat is believed to exert a more deleterious effect on beta cells than unsaturated fat, this is mostly based on in vitro data because, until recently, palmitate infusion has been a challenge [38].

Salicylate has previously been found to restore glucose-stimulated insulin secretion in an in vitro glucotoxicity model [7]. Although not all studies are concordant [39], the majority report that salicylate improves beta cell function in humans [40–42]. This effect was initially attributed to inhibition of COX-2 [32], the gene for which is controlled by NFκB, and the consequently decreased synthesis of PGE2, a prostaglandin which inhibits insulin secretion. In addition to inhibiting *Cox2* (also known as *Ptgs2*) transcription via NFκB, salicylate is a direct inhibitor of both COX-1 and COX-2 [43], and an activator of AMPK [29]. Salicylate did not likely protect

against beta cell dysfunction through COX-1 inhibition as a COX-1 inhibitor did not prevent oleate-induced beta cell dysfunction. AMPK phosphorylation, however, did tend to decrease with oleate and this decrease was prevented by salicylate, which raises the question as to whether the protective effect of salicylate was mediated in part by AMPK activation. The effect of AMPK on insulin secretion is generally considered to be inhibitory [44]; however, AMPK may also deplete islet fat [45] and inhibit IKKβ [30]. To further implicate IKKβ in the effect of oleate to decrease beta cell function, we also used BMS, an inhibitor that, to our knowledge, has not been reported to activate AMPK; we obtained the same results for beta cell function as with salicylate both in vitro and in vivo. Importantly, the effect of IKKβ to mediate fat-induced beta cell dysfunction is also supported by our ex vivo and in vivo data using a genetic inhibition model, the *Ikkb*$^{\Delta\text{beta cell}}$ mouse.

In mice, however, two important differences from the rat studies were noted. First, 48 h exposure to oleate induced marked insulin resistance in mice in accordance with our previously published data [20]. Besides species difference, the reason behind this finding may be the strain of mice, as C57BL/6 mice are very susceptible to fat-induced insulin resistance [46]. Oleate infusion, which induced insulin resistance in mice, did not result in lower absolute insulin secretion during the clamp, but decreased beta cell function (DI), similar to our results with olive oil in rats. Second, BMS did restore DI but did not affect oleate-induced insulin resistance, as it likely effected a decrease in insulin sensitivity itself, which resulted in increased plasma insulin and C-peptide during clamps. Decreased insulin sensitivity with an IKKβ inhibitor may be dose related and due to the inhibition of COX-2-derived prostaglandins that increase insulin action [47].

The mechanisms whereby IKKβ inhibition prevents fat-induced beta cell dysfunction deserve further study. However, both upregulation of insulin signalling and COX-2 inhibition

are plausible mechanisms, as suggested by two lines of evidence. First, salicylate prevented serine phosphorylation of IRS, which is known to decrease insulin-induced tyrosine phosphorylation (i.e. insulin signalling). Beta cell insulin signalling is known to be important for beta cell function [48]. Second, salicylate prevented the oleate-induced PGE2 production and a COX-2 inhibitor mimicked the effect of salicylate. Previously, the effect of COX-2 on beta cell function has been studied mainly in the context of cytokine exposure, with contrasting results, presumably due to COX-1 vs COX-2 specificity of the inhibitors used [32,49]. Also, the effects of exposure to COX-2 products yielded variable results among laboratories [49,50] but a dose-dependent inhibitory effect [49,50] suggests COX-2 may be implicated in decreasing beta cell function. As oleate increased *Il1ra* (also known as *Il1rn*) gene expression to a greater extent than that of *Il1b*, the increase in *Cox2* mRNA expression is likely due to an oxidative stress-induced activation of IKKβ, independent of IL-1β. Nonetheless, salicylate did prevent upregulated gene expression of cytokines and chemokines, which may have contributed to beta cell dysfunction by further activating IKKβ and possibly other inflammatory pathways.

In summary, we demonstrated that prolonged exposure to fatty acids, which induces oxidative stress in islets, decreases beta cell function both in vitro and in vivo via activation of IKKβ. The novelty of our findings is the demonstration that IKKβ mediates beta cell dysfunction induced by NEFA selectively, and that the IKKβ/NFκB pathway is a therapeutic target to prevent NEFA-induced beta cell dysfunction in vivo.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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Table 1 Plasma NEFA, triacylglycerol, glucose and insulin levels after 48 h infusions

Treatment	NEFA (mmol/l)	Triacylglycerol (mmol/l)	Glucose (mmol/l)	Insulin (pmol/l)
Saline (n=12)	0.693 ± 0.026	0.17±0.019	5.8±0.2	79±12
Oleate (n=10)	1.050±0.118**	0.21±0.014	5.9±0.1	72±13
OLO (n=7)	0.925±0.104*	0.60±0.063***	5.5±0.2	110±80
Oleate + SLY (n=8)	1.123±0.168**	0.25±0.020	5.2±0.1	68±14
OLO + SLY (n=11)	1.027±0.095*	0.54±0.057***	5.0±0.1	82±15
SLY (n=9)	0.580±0.095	0.15±0.024	5.4±0.1	57±90

Data are mean ± SEM

Rats were treated with: saline; oleate at 1.3 μmol/min; OLO (20% olive oil infusate containing 50 U/ml heparin) at 5.5 μl/min; oleate + salicylate at 0.7 μmol kg^{-1} min^{-1}; OLO + salicylate; or salicylate only

*p<0.05, **p<0.01 and ***p<0.001 compared with saline and salicylate only

SLY, salicylate

Table 2 mRNA levels of inflammatory markers in freshly isolated islets of infused rats

Marker	Saline	Oleate	Oleate + SLY	SLY
Il1b	0.82 ± 0.17 (n=8)	2.02 ± 0.42††,‡‡‡,§§§ (n=8)	0.30 ± 0.12 (n=5)	0.10 ± 0.04† (n=3)
Tnfa (*Tnf*)	0.58 ± 0.22 (n=6)	1.97 ± 0.28†,§§ (n=7)	0.81 ± 0.46 (n=4)	0.08 ± 0.04 (n=3)
Mcp1 (*Ccl2*)	0.76 ± 0.28 (n=6)	5.64 ± 1.89††,‡‡‡,§§§ (n=10)	0.52 ± 0.24 (n=5)	0.01 ± 0.00† (n=3)
Il1ra (*Il1rn*)	1.00 ± 0.31 (n=5)	15.52 ± 4.67†† (n=10)	2.79 ± 1.34 (n=4)	4.70 ± 2.79 (n=3)
Tgfb (*Tgfb1*)	0.85 ± 0.26 (n=7)	2.47 ± 0.51†,‡,§§ (n=7)	0.81 ± 0.37 (n=4)	0.54 ± 0.33 (n=4)
Cd68	0.62 ± 0.14 (n=8)	4.21 ± 1.07†††,‡‡,§§ (n=7)	0.54 ± 0.12 (n=4)	0.75 ± 0.37 (n=3)
Cox2 (*Ptgs2*)	1.00 ± 0.10 (n=5)	2.66 ± 0.53††,‡‡‡,§§§ (n=9)	0.36 ± 0.10† (n=3)	0.33 ± 0.09† (n=4)

Data are mean ± SEM

Rats were infused with: saline; oleate; oleate + salicylate; or salicylate only

Units are normalised to a housekeeping gene

Sample size differed between markers as some samples had expression levels that were either undetectable or outliers according to the Grubb's test

$^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ and $^{\dagger\dagger\dagger}p<0.001$ compared to saline

$^{\ddagger}p<0.05$, $^{\ddagger\ddagger}p<0.01$ and $^{\ddagger\ddagger\ddagger}p<0.001$ compared to oleate + salicylate

$^{\S}p<0.05$, $^{\S\S}p<0.01$ and $^{\S\S\S}p<0.001$ compared to salicylate alone

SLY, salicylate

Figure Legends

Fig. 1 Plasma glucose (**a**, **b**), GINF (**c**, **d**), insulin (**e**, **f**) and C-peptide (**g**, **h**) during two-step hyperglycaemic clamps following 48 h of oleate or OLO infusion. (**a**, **c**, **e**, **g**) Rats were treated with: saline *n*=12; oleate at 1.3 μmol/min (*n*=10); oleate + salicylate at 0.7 μmol kg^{-1} min^{-1} (*n*=8); or salicylate only (*n*=9). (**b**, **d**, **f**, **h**) Rats were treated with: saline (*n*=12); OLO (20% olive oil infusate containing 50 U/ml heparin) at 5.5 μl/min (*n*=7); OLO + salicylate (*n*=11); or salicylate only (*n*=9). Data are mean ± SEM. ***p*<0.01 and ****p*<0.001 vs all, throughout the clamp. White circles, saline; black circles, oleate; black triangles, oleate + salicylate; white triangles, salicylate; black squares, OLO; black diamonds, OLO + salicylate

Fig. 2 *M*/I and DI during two-step hyperglycaemic clamps with and without infusion for 48 h of oleate (**a**, **c**) or OLO (**b**, **d**). See ESM Methods for calculation of these indices. The groups are as described for Fig. 1. Data are mean ± SEM. ***p*<0.01 vs all groups at the same glucose concentration. White bars, saline; light grey bars, salicylate only. In (**a**, **c**): black bars, oleate; dark grey bars, oleate + salicylate. In (**b**, **d**): diagonal striped bars, OLO; horizontal striped bars, OLO + salicylate

Fig. 3 Insulin secretory response to glucose (**a**, **b**), and quantification of phosphorylated IkBα (**c**, **d**) and active nuclear NFκB (**e**, **f**) in freshly isolated islets from fat-infused rats. Groups are as described for Fig. 1. In (**a**, **b**): saline, *n*=16; oleate, *n*=14; oleate + salicylate, *n*=8; OLO, *n*=12; OLO + salicylate, *n*=6; salicylate only, *n*=10. In (**c**–**f**): saline, *n*=5–7; oleate, *n*=4–6; oleate + salicylate, *n*=4–5; OLO, *n*=5–6; OLO + salicylate, *n*=5–8; salicylate only, *n*=4–6. Data are mean ± SEM. **p*<0.05, ***p*<0.01 and ****p*<0.001 vs all (in **a** and **b**, comparison is to all groups at the same glucose concentration). White bars, saline; light grey bars, salicylate. In (**a**, **c**, **e**): black

bars, oleate; dark grey bars, oleate + salicylate. In (**b**, **d**, **f**): diagonal striped bars, OLO; horizontal striped bars, OLO + salicylate

Fig. 4 Plasma glucose (**a**), GINF (**b**), plasma insulin (**c**), C-peptide (**d**), *M*/I (**e**) and DI (**f**) during hyperglycaemic clamps in mice treated for 48 h with: saline (*n*=7); oleate, 0.4 μmol/min (*n*=6); oleate + BMS, 0.12 mmol kg^{-1} day^{-1} (*n*=5); or BMS only (*n*=3). Data are mean ± SEM. $^{**}p<0.01$ vs all; $^{\dagger}p<0.05$ vs saline; $^{\ddagger}p<0.05$ vs oleate. White circles/bars, saline; black circles/bars, oleate; black triangles/dark grey bars, oleate + BMS; white triangles/light grey bars, BMS only

Fig. 5 IKKβ protein levels in islets (**a**) and hypothalamus (**b**) of $Ikkb^{\Delta\text{beta cell}}$ mice ($Ikkb^{F/F}$:RIP2-Cre) and floxed controls ($Ikkb^{F/F}$). Each lane represents islets pooled from two mice of the same genotype (**a**) or one hypothalamus taken from an individual mouse (**b**). Plasma NEFA (**c**) and insulin secretory response to glucose of freshly isolated islets of $Ikkb^{F/F}$ and $Ikkb^{\Delta\text{beta cell}}$ mice (**d**) following 48 h oleate (0.4 μmol/min) or saline infusion. Control/saline, *n*=8; control/oleate, *n*=7; $Ikkb^{\Delta\text{beta cell}}$/oleate, *n*=5; $Ikkb^{\Delta\text{beta cell}}$/saline, *n*=9. Data are mean ± SEM. $^{\dagger}p<0.05$ vs control/saline. White bars, control/saline; black bars, control/oleate; dark grey bars, $Ikkb^{\Delta\text{beta cell}}$/oleate; light grey bars, $Ikkb^{\Delta\text{beta cell}}$/saline

Fig. 6 NEFA levels prior to hyperglycaemic clamp (**a**), *M*/I (**b**) and DI (**c**) during hyperglycaemic clamps in $Ikkb^{F/F}$ (control) and $Ikkb^{\Delta\text{beta cell}}$ mice following 48 h oleate (0.4 μmol/min) or saline infusion. Control/saline, *n*=8; control/oleate, *n*=7; $Ikkb^{\Delta\text{beta cell}}$/oleate, *n*=8; $Ikkb^{\Delta\text{beta cell}}$/saline, *n*=5. Data are mean ± SEM. $^{*}p<0.05$ vs. control/saline and $Ikkb^{\Delta\text{beta cell}}$/saline; $^{\dagger\dagger}p<0.01$ vs. control/saline; $^{\ddagger}p<0.05$ and $^{\ddagger\ddagger}p<0.01$ vs. $Ikkb^{\Delta\text{beta cell}}$/oleate. White bars, control/saline; black bars, control/oleate; dark grey bars, $Ikkb^{\Delta\text{beta cell}}$/oleate; light grey bars, $Ikkb^{\Delta\text{beta cell}}$/saline

Fig. 7 Levels of insulin secretion (**a**), IκBα (**b**) and phosphorylated AMPKα level (**c**) in islets exposed for 48 h to control or oleate with/without salicylate. Control, 0.5% NEFA-free BSA in medium: (**a**) *n*=7, (**b**) *n*=7, (**c**) *n*=8. Oleate, 0.4 mmol/l in 0.5% NEFA-free BSA: (**a**) *n*=14, (**b**) *n*=8, (**c**) *n*=7. Oleate + salicylate, 0.25 mmol/l: (**a**) *n*=10, (**b**) *n*=7, (**c**) *n*=8. Salicylate only, in BSA: (**a**) *n*=7, (**b**) *n*=4, (**c**) *n*=6. (**d**) Insulin secretion in islets exposed to oleate for 48 h with/without BMS. Control, *n*=7; oleate, *n*=14; oleate + BMS, 3 μmol/l, *n*=10; BMS only, in BSA, *n*=7. Data are mean ± SEM. $^{**}p<0.01$ vs all; $^{\dagger}p<0.05$ vs control. White bars, control; black bars, oleate. In (**a**–**c**): dark grey bars, oleate + salicylate; light grey bars, salicylate. In (**d**): dark grey bars, oleate + BMS; light grey bars, BMS. CON, control; OLE, oleate; SLY, salicylate

Fig. 8 Serine phosphorylated IRS-1 (**a**) and PGE2 levels (**b**) in cultured islets exposed for 48 h to oleate or vehicle control with/without salicylate. Control, 0.5% NEFA-free BSA in medium: (**a**) *n*=5, (**b**) *n*=5. Oleate, 0.4 mmol/l in 0.5% NEFA-free BSA: (**a**) *n*=5, (**b**) *n*=5. Oleate + salicylate, 0.25 mmol/l: (**a**) *n*=5, (**b**) *n*=6. Salicylate only, in BSA: (**a**) *n*=5, (**b**) *n*=5. (**c**) Insulin secretion in control or oleate with/without the COX-2 inhibitor SC-236 (10 μmol/l). Control, *n*=11; oleate, *n*=10; oleate + SC-236, *n*=9; SC-236 only, in BSA, *n*=9. Data are mean ± SEM. $^{*}p<0.05$ and $^{**}p<0.01$ vs all; $^{\dagger}p<0.05$ vs control. White bars, control; black bars, oleate. In (**a**, **b**): dark grey bar, oleate + salicylate; light grey bars, salicylate. In (**c**): dark grey bars, oleate + SC-236; light grey bars, SC-236. CON, control; OLE, oleate; SLY, salicylate

Fig. 1

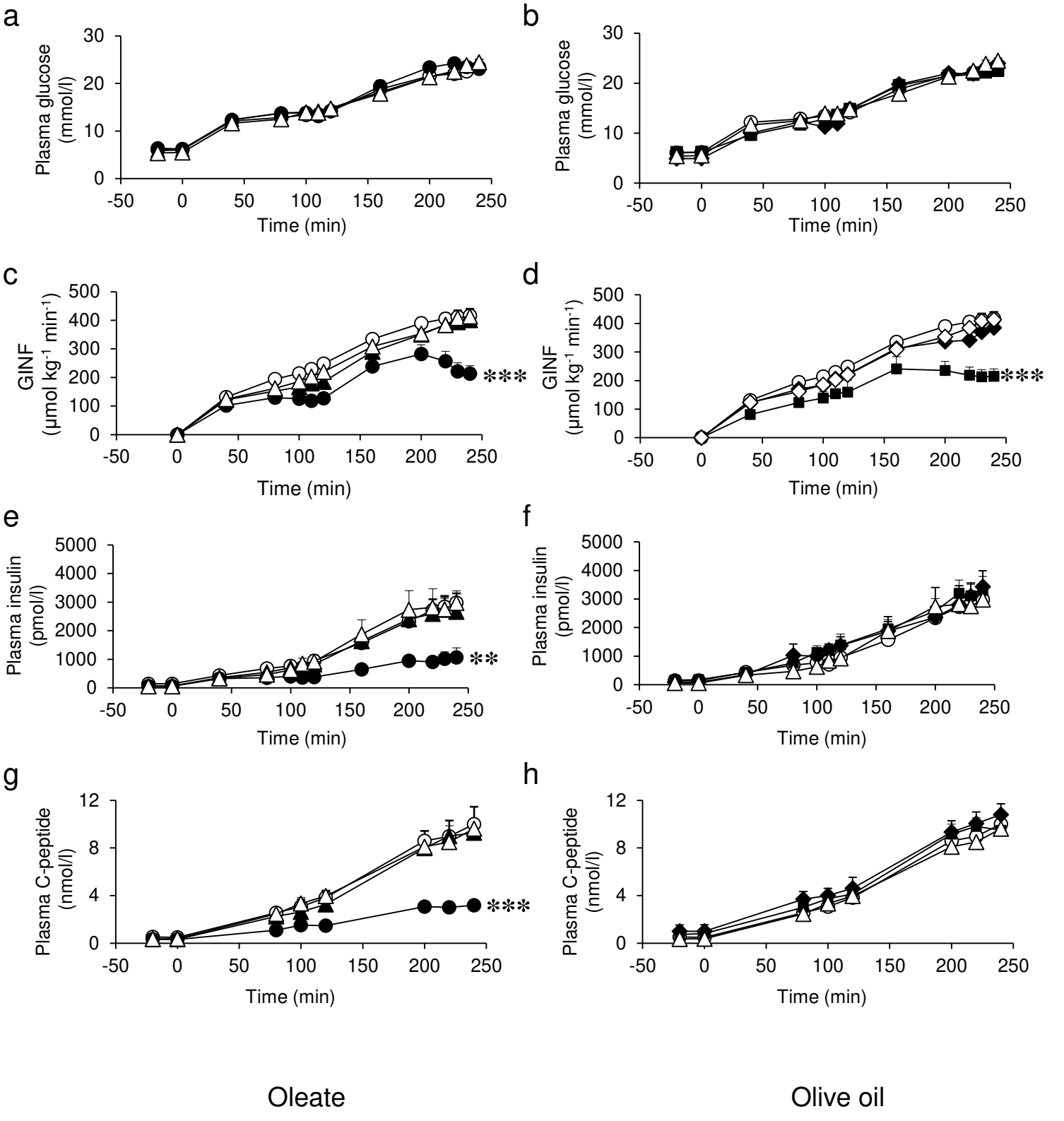

a
b
c
d
e
f
g
h
Plasma glucose (mmol/l)
GINF (µmol kg-1 min-1)
Plasma insulin (pmol/l)
Plasma C-peptide (nmol/l)
Time (min)
***
**
Oleate
Olive oil

Fig. 2

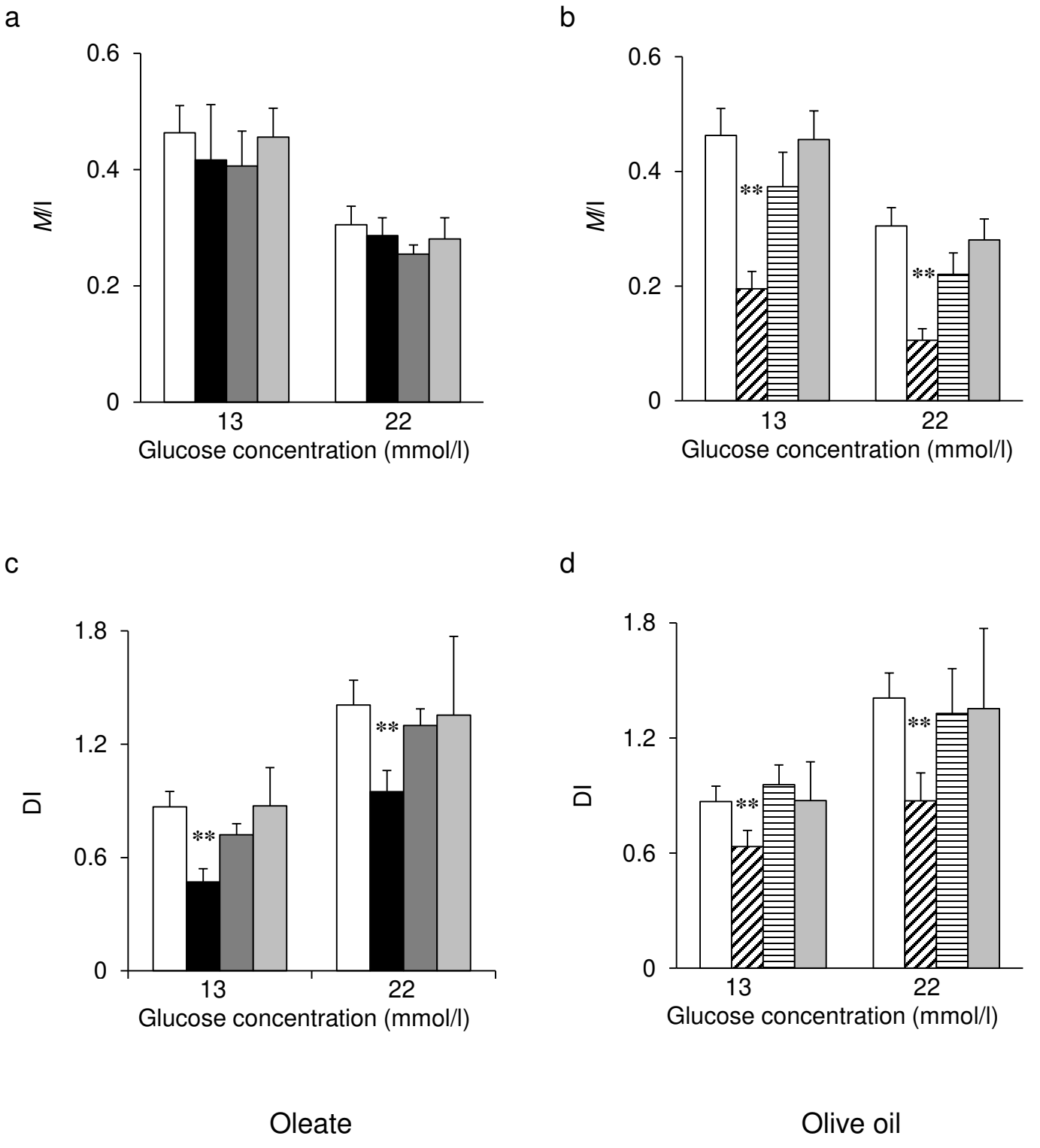

a
b
c
d
M/I
DI
0.6
0.4
0.2
0
1.8
1.2
0.6
0
13
22
Glucose concentration (mmol/l)
**
Oleate
Olive oil

Fig. 3

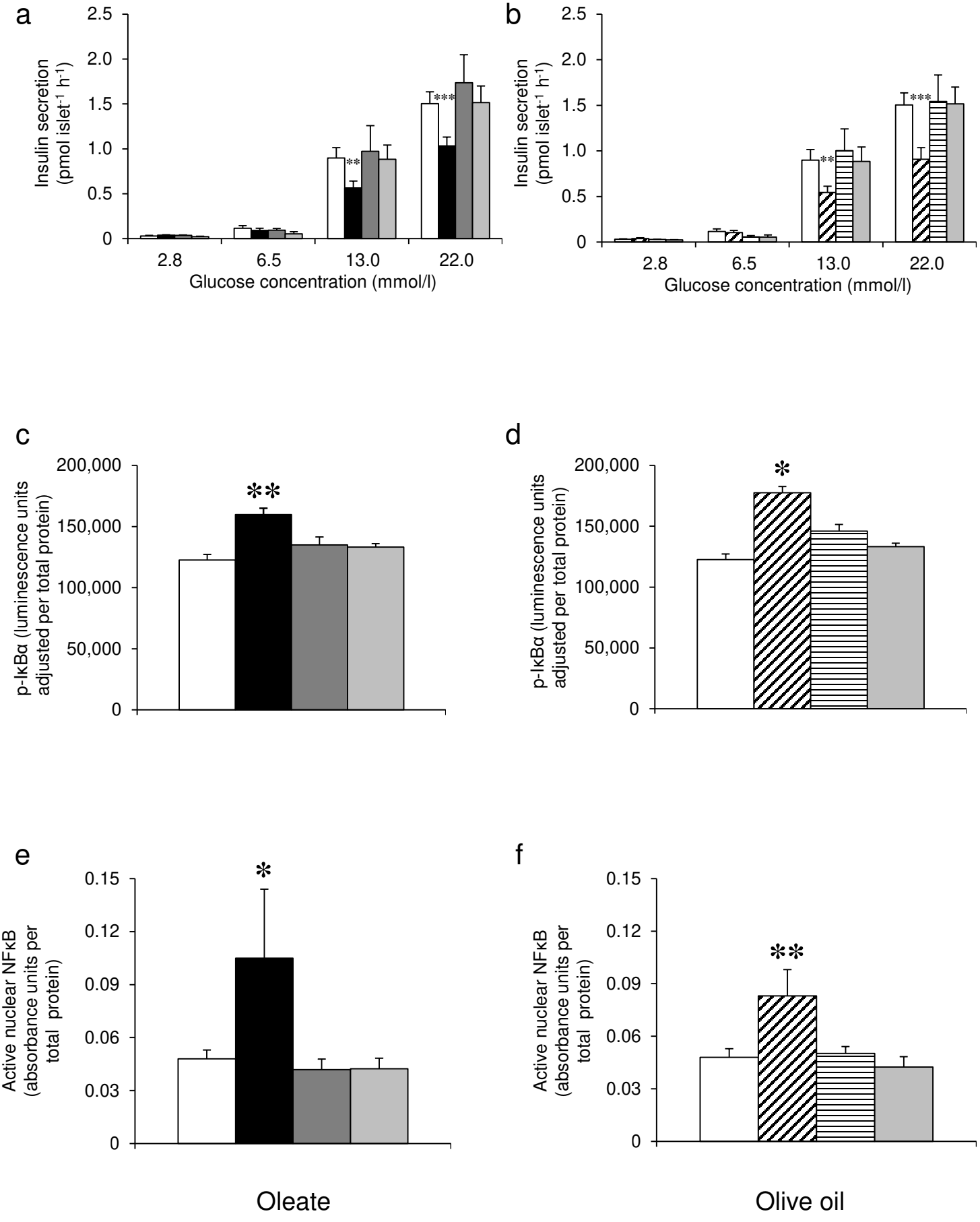

a
b
Insulin secretion (pmol islet-1 h-1)
2.5
2.0
1.5
1.0
0.5
0
**
***
2.8
6.5
13.0
22.0
Glucose concentration (mmol/l)
c
d
p-IκBα (luminescence units adjusted per total protein)
200,000
150,000
100,000
50,000
0
**
*
e
f
Active nuclear NFκB (absorbance units per total protein)
0.15
0.12
0.09
0.06
0.03
0
*
**
Oleate
Olive oil

Fig. 4

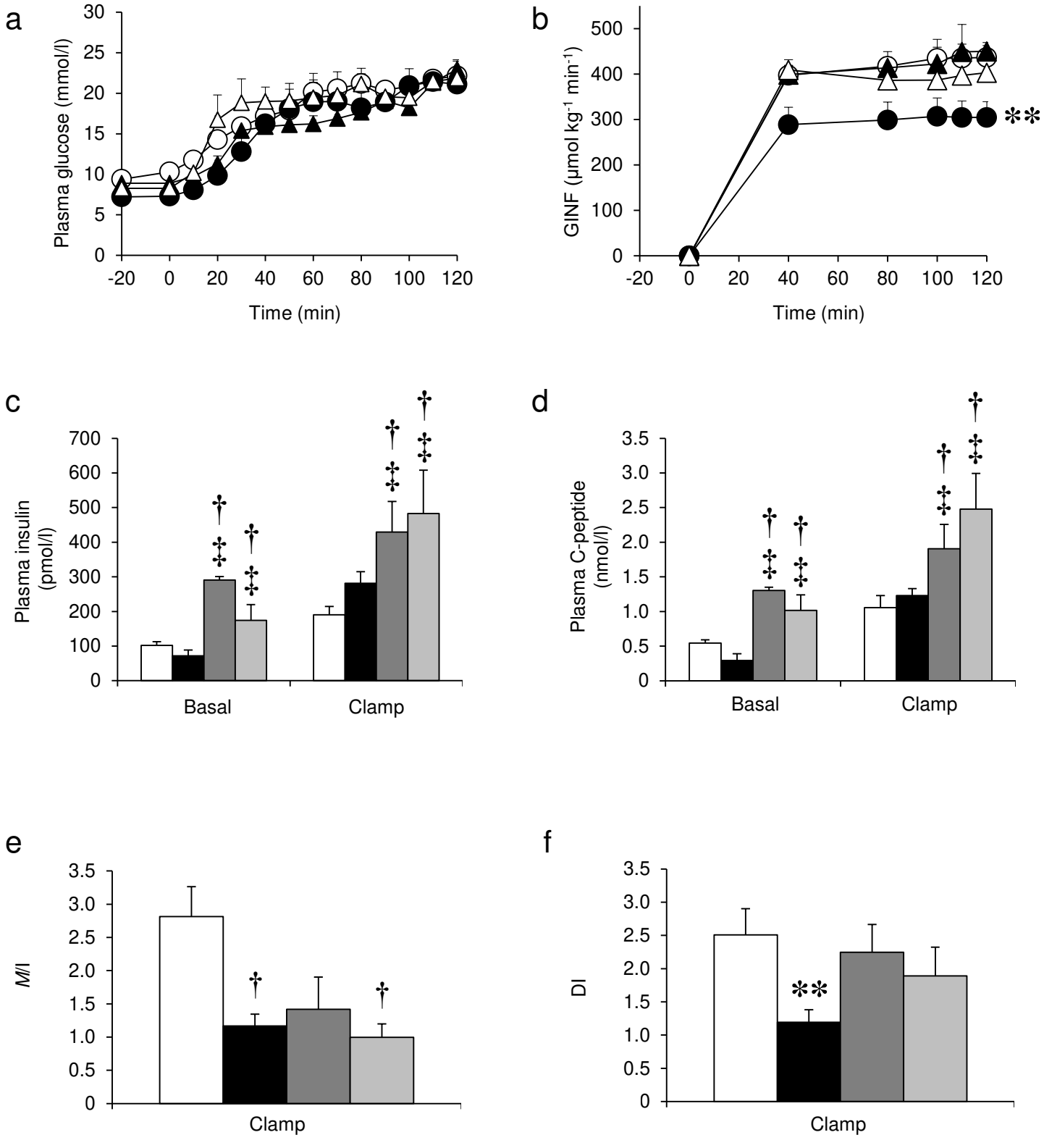
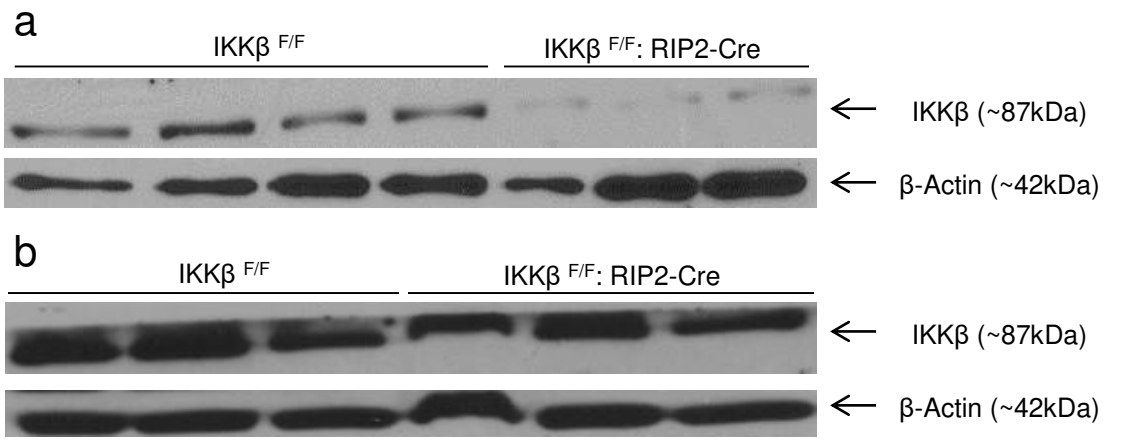
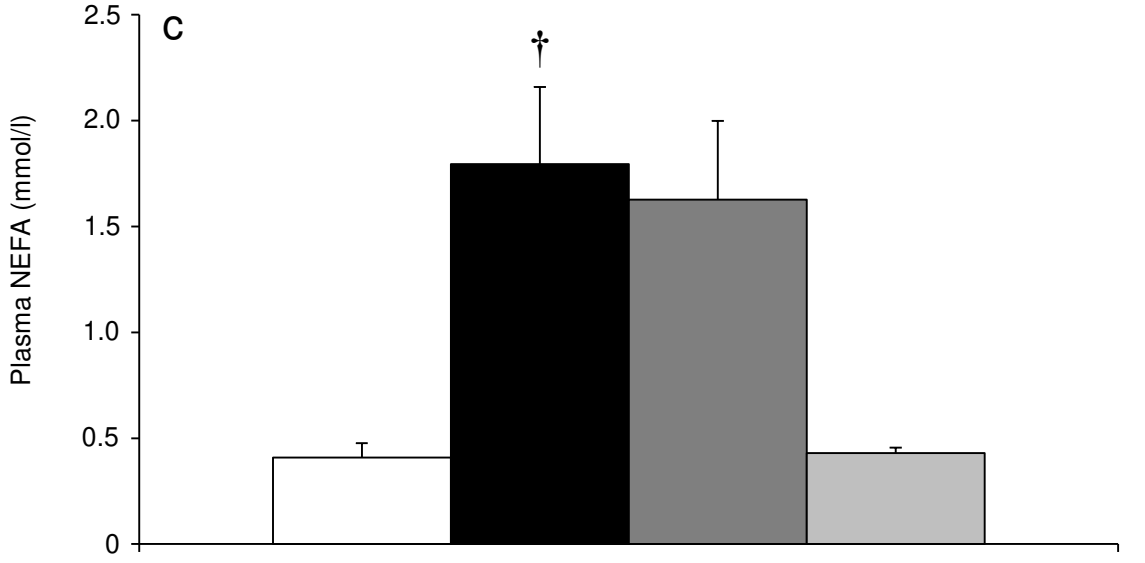

a
Plasma glucose (mmol/l)
Time (min)
b
GINF (μmol kg-1 min-1)
Time (min)
**
c
Plasma insulin (pmol/l)
Basal
Clamp
d
Plasma C-peptide (nmol/l)
Basal
Clamp
e
M/I
Clamp
f
DI
**
Clamp

Fig. 5


a
IKKβ F/F
IKKβ F/F: RIP2-Cre
IKKβ (~87kDa)
β-Actin (~42kDa)
b
IKKβ F/F
IKKβ F/F: RIP2-Cre
IKKβ (~87kDa)
β-Actin (~42kDa)


c
Plasma NEFA (mmol/l)
2.5
2.0
1.5
1.0
0.5
0
†

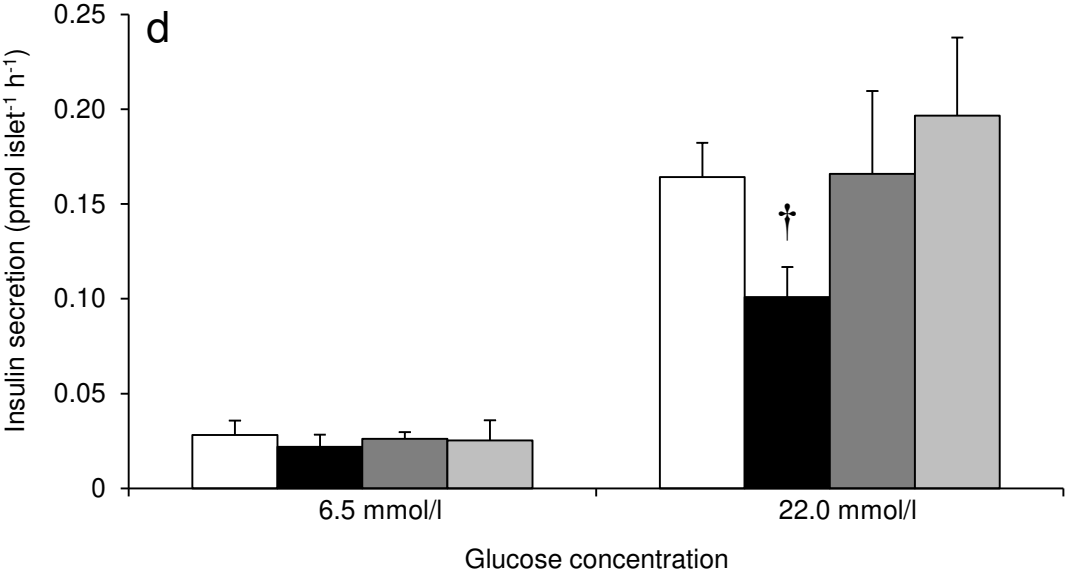

d
Insulin secretion (pmol islet-1 h-1)
0.25
0.20
0.15
0.10
0.05
0
†
6.5 mmol/l
22.0 mmol/l
Glucose concentration

Fig. 6

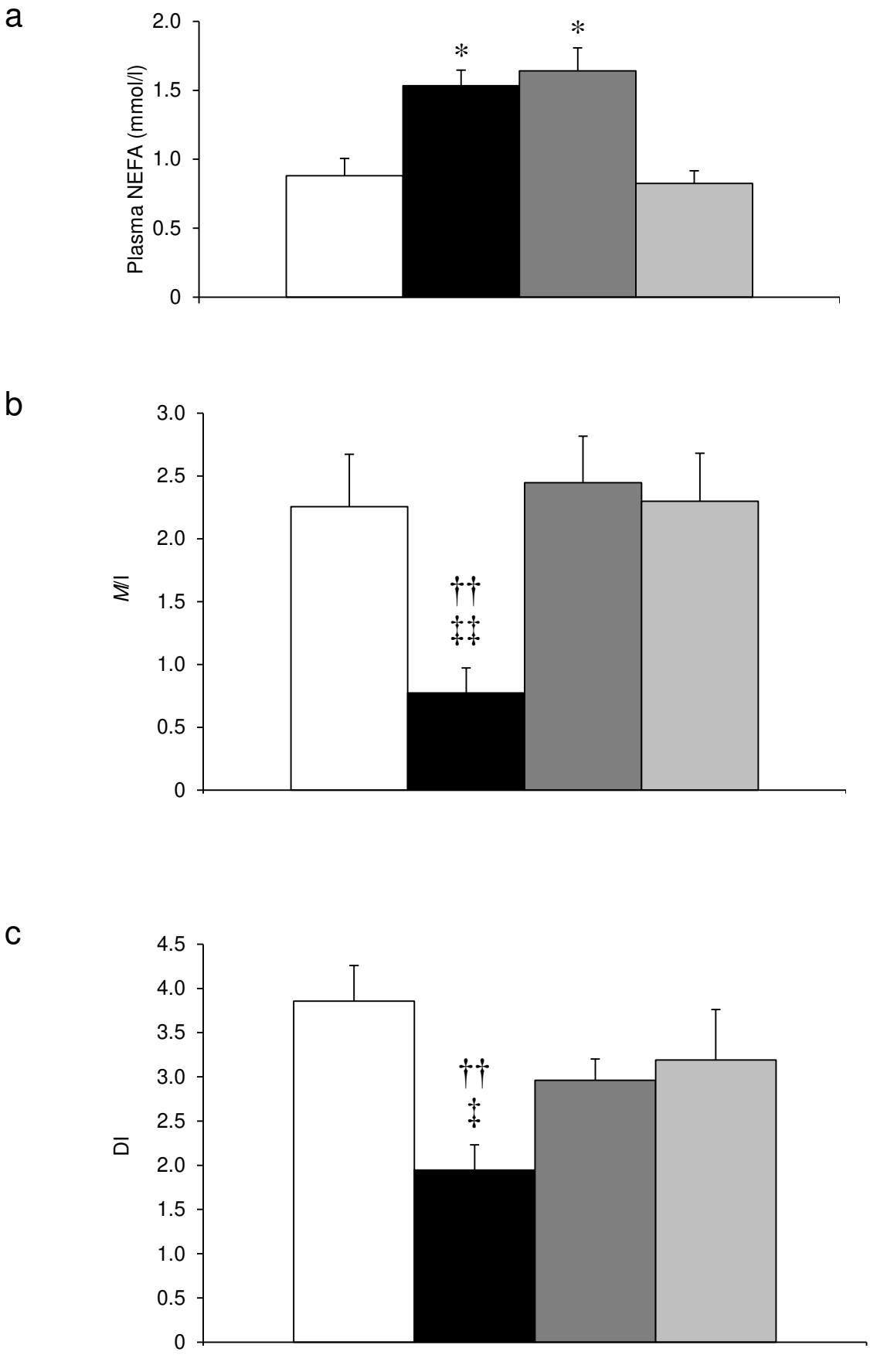

a
Plasma NEFA (mmol/l)
2.0
1.5
1.0
0.5
0
*
*
b
M/I
3.0
2.5
2.0
1.5
1.0
0.5
0
††
‡‡
c
DI
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
††
‡

Fig. 7

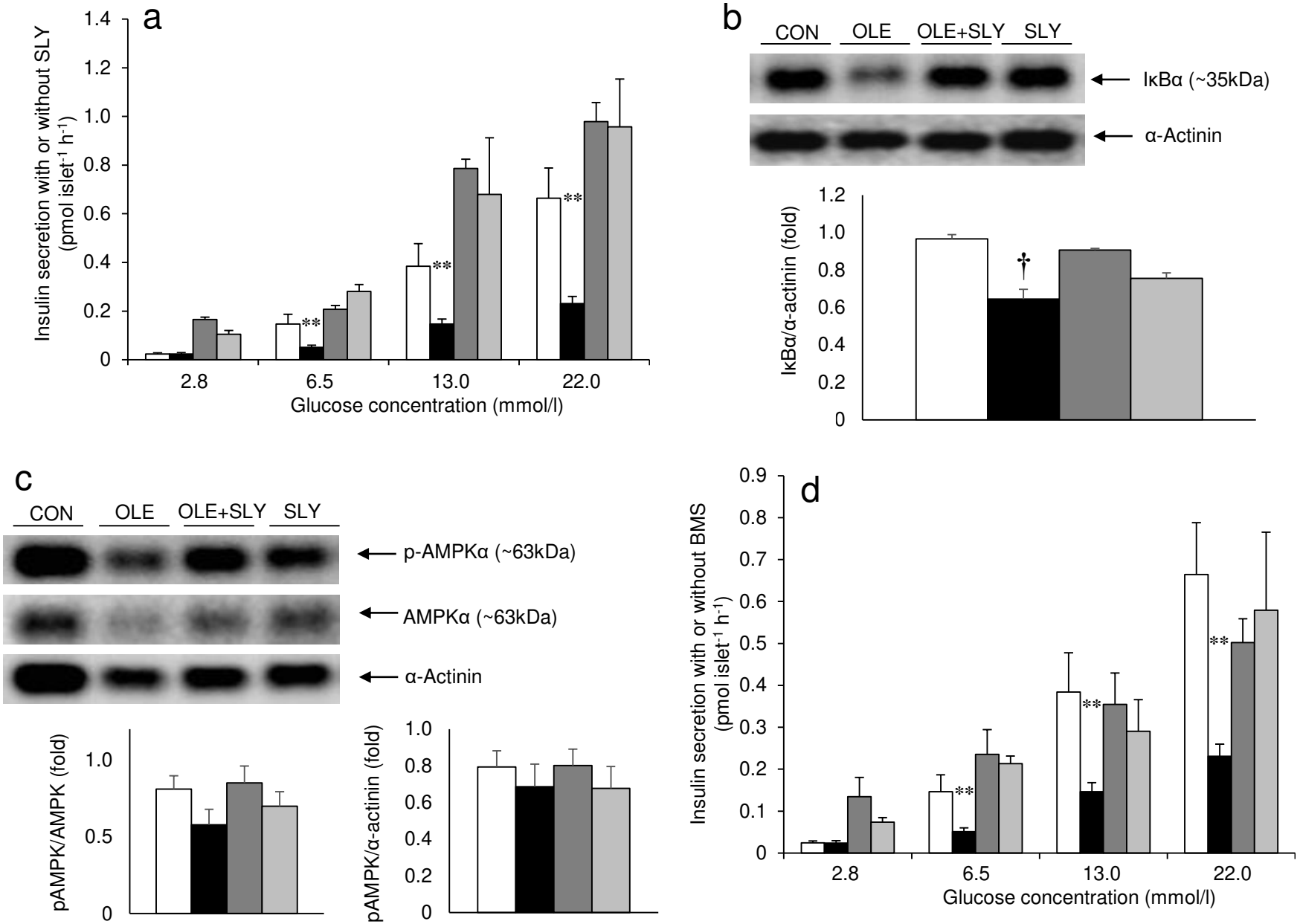
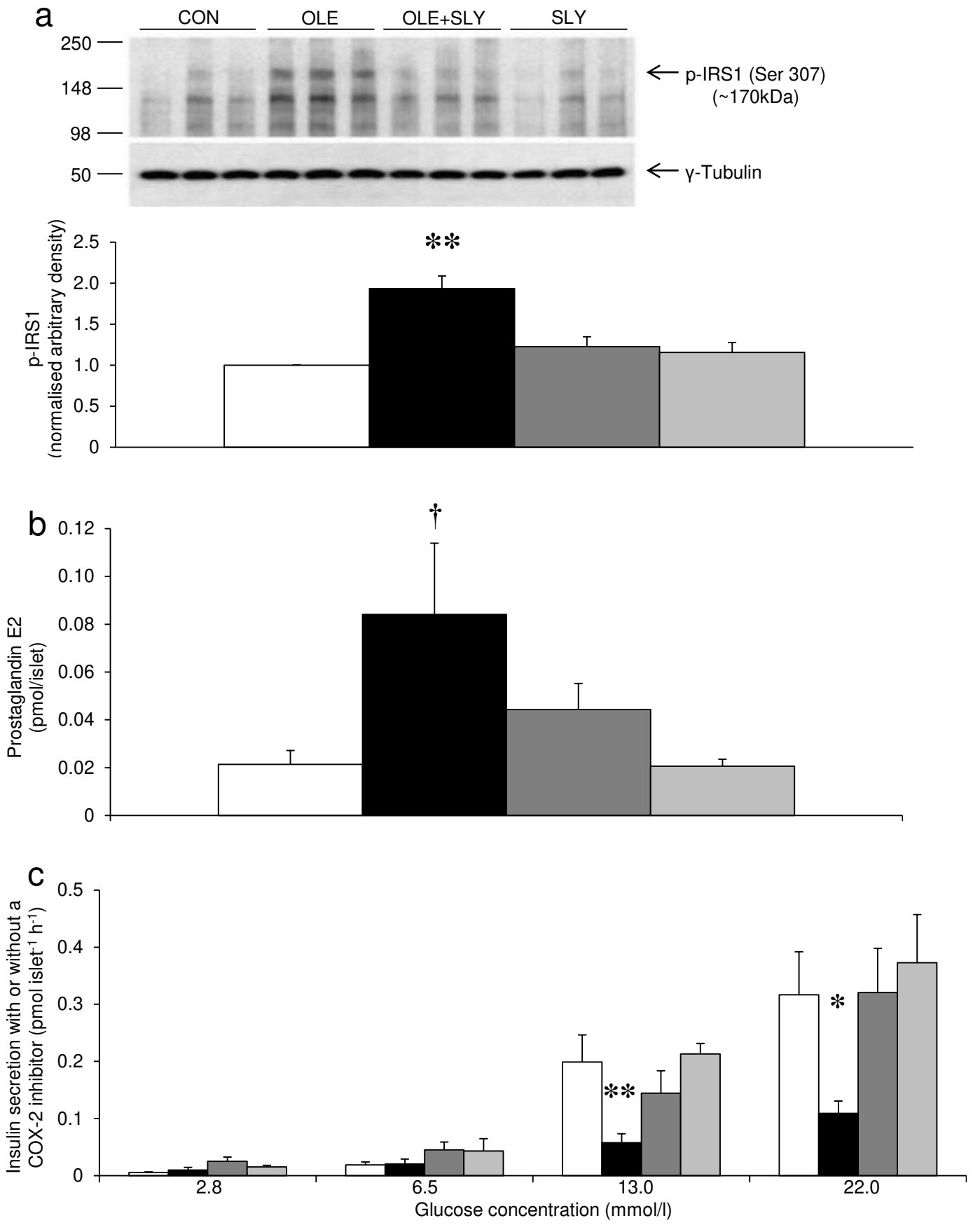

a
Insulin secretion with or without SLY (pmol islet-1 h-1)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
**
2.8
6.5
13.0
22.0
Glucose concentration (mmol/l)
b
CON
OLE
OLE+SLY
SLY
IκBα (~35kDa)
α-Actinin
IκBα/α-actinin (fold)
1.2
1.0
0.8
0.6
0.4
0.2
0
†
c
CON
OLE
OLE+SLY
SLY
p-AMPKα (~63kDa)
AMPKα (~63kDa)
α-Actinin
pAMPK/AMPK (fold)
1.0
0.5
0
pAMPK/α-actinin (fold)
1.0
0.8
0.6
0.4
0.2
0
d
Insulin secretion with or without BMS (pmol islet-1 h-1)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
**
**
**
2.8
6.5
13.0
22.0
Glucose concentration (mmol/l)

Fig. 8


a
CON
OLE
OLE+SLY
SLY
250
148
98
50
← p-IRS1 (Ser 307)
(~170kDa)
← γ-Tubulin
p-IRS1
(normalised arbitrary density)
2.5
2.0
1.5
1.0
0.5
0
**
b
Prostaglandin E2
(pmol/islet)
0.12
0.10
0.08
0.06
0.04
0.02
0
†
c
Insulin secretion with or without a
COX-2 inhibitor (pmol islet-1 h-1)
0.5
0.4
0.3
0.2
0.1
0
**
*
2.8
6.5
13.0
22.0
Glucose concentration (mmol/l)

IKKβ inhibition prevents fat-induced beta cell dysfunction in vitro and in vivo in rodents

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Methods

Animals

Animals were housed in rodent microisolator cages with corn cob bedding in the University of Toronto's Department of Comparative Medicine (DCM) in a temperature (21^{o} C) and humidity (40-60%)-controlled room within a specific pathogen free facility. They were exposed to a 12 h light/dark cycle and were allowed free access to water and rodent chow (Teklad Global 2018, Madison, WI, USA). Environmental enrichment was provided according to DCM and animals were frequently handled to minimise stress. All animals used in experiments were deemed healthy by the DCM prior to surgery as well as post-surgery and prior to treatment. The invasive nature of the surgery and experimental protocol leads to a dropout rate of ~20%, primarily due to stroke resulting from clotting at the catheter site. Sterile technique and the lowest effective treatment doses were used to ensure the dropout rate would not be greater than this and all animals that experienced adverse events were excluded from experiments. Animals were monitored and given analgesic (buprenorphine – mice: 0.21 μmol/kg, rats: 0.11 μmol/kg) during surgery and at least every 12 h after surgery for 48 hours. Rats were housed in pairs prior to surgery and

individually for recovery and experiments. For experiments, they were placed in metabolic cages. They were ordered from Charles River prior to experiments. Mice were bred in the DCM and prior to surgery there were 1-4 mice per cage, depending on the size of the litter. Following surgery and during experiments, mice were housed individually. At the end of experiments (clamps or infusions for islet isolation), tissues were obtained under deep anaesthesia (cocktail of ketamine:xylazine:acepromazine, 370:8:1.2 mmol/l, 2.5 μl/g in mice, i.p.; 1.5 μl/g in rats, i.p.) followed by euthanasia via cocktail overdose (1ml/rat, 0.2ml/mouse, i.p.).

Sample size

The number of animals and samples to be used in each experiment was determined using power calculation with a power >80%. Plasma assays (insulin, C-peptide, NEFA and triacylglycerol) were performed in duplicate and the averages are reported. Glucose-stimulated insulin secretion studies in rat islets were performed in triplicate and in mice islets were performed in duplicate and the averages are reported.

Evaluation of insulin sensitivity and beta cell function

Insulin sensitivity index The insulin sensitivity index *M*/I, where *M* stands for glucose metabolism and I = plasma insulin [1] was calculated during the last 40 minutes of each step of the two step hyperglycaemic clamp in rats and during the last 20 minutes of the one-step hyperglycaemic clamp in mice according to the following formula: *M*/I=GINF/Insulin where GINF is the rate of glucose infusion and Insulin is the plasma insulin concentration. The unit is $\mu mol\,kg^{-1}\,min^{-1}$ GINF divided by pmol/l insulin. Although there are limitations to assessing insulin sensitivity during hyperglycaemic

clamps [2], it is too invasive to perform additional hyperinsulinaemic-euglycaemic clamps (the gold-standard method to assess insulin sensitivity) in the same small animal.

Disposition index The disposition index (DI), which is an established index [3] of beta cell function based on the hyperbolic relationship between insulin sensitivity and insulin secretion [4], was calculated during the last 40 minutes in rats and the last 20 minutes in mice as DI=$M/\mathrm{I} \times$ C-peptide. The unit is $\mu\mathrm{mol\,kg^{-1}\,min^{-1}}$ GINF divided by pmol/l insulin multiplied by nmol/l C-peptide. C-peptide was used as an index of absolute insulin secretion because insulin secretion rate cannot be calculated in rats or mice due to the unknown kinetics of C-peptide (as species-specific C-peptide is unavailable for injection). We have previously validated this index in rodents [5-7].

Determinations in islets of rats infused with oleate/control with/without salicylate

Phosphorylated IκBα assay Whole islet-extract was obtained according to manufacturer's protocol. Protein concentration was determined using the Pierce bicinchoninic acid (BCA) protein assay. Islet-lysates were assayed for phosphorylated IκBα by an ELISA kit previously used by our group [8]. The absorbance levels were in the upper detection range of the standard curve. The intra- and inter-assay coefficients of variations are <10% and <20%, respectively (Active Motif, Carlsbad, CA, USA).

NFκB p65 activity assay Nuclear proteins were extracted according to the manufacturer's instructions. The supernatant (nuclear fraction) was kept at -80°C until analysis. The active NFκB contained in the nuclear extracts was measured by its DNA binding activity on immobilized oligonucleotides encoding a specific consensus site using a NFκB p65 transcription factor ELISA kit previously used by our group [8]. We used greater than 10 times more protein than the detection limit (0.5µg nuclear extract/well) and the intra- and

inter-assay coefficients of variations are <10% and <20%, respectively (Active Motif, Carlsbad, CA).

ROS measurements Islets were incubated with 10 μmol/l dihydro-dichlorofluorescein-diacetate (H2DCF-DA) (D6883; Sigma) in KRBH containing 2.8 mmol/l glucose for 30 minutes [9]. The medium was then replaced with fresh KRBH containing no glucose, and fluorescence was measured at 480 nm excitation and 510 nm emission with an Olympus microscope. Data were analysed using ImageMaster3.

Real-time RT-PCR ESM Table 1 reports the primers used for each gene.

Western blots

Western blots were performed in cultured rat islets and in freshly isolated mouse islets (IKKβ). Islet pellets (100~150 islets per sample) were lysed in RIPA buffer (50 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) supplemented with 1 mmol/l phenylmethane sulfonyl fluoride (PMSF), 1.5 nmol/ml aprotinin, 23 nmol/ml leupeptin, and incubated on ice for 30 min. The samples were then centrifuged at 13,000 rpm, 4°C for 10 min. The supernatant was removed and the protein concentration was measured as described above or by Bradford assay (BioRad, Mississauga, ON, Canada). 30 μg of protein from each islet lysate was resolved by SDS-PAGE, transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membranes and immunoblotted with the antibodies described in the main text. All antibodies, except IKKβ, have been previously validated in published studies by the laboratory [10-13]. Secondary anti-mouse (Cell Signaling Technology, 7076, raised in horse, RRID:AB_330924, 1:10,000) and anti-rabbit (Cell Signaling Technology, 7074, raised in goat, RRID:AB_2099233, 1:10,000) IgG antibodies conjugated to horse radish

peroxidase and enhanced chemiluminescence system (Amersham Biosciences, Quebec, QC, Canada) were used for detection. For phospho-Thr172-AMPKα, AMPKα, IκBα and α-actinin Kodak Imager 4000pro (Carestream, USA) was used to image membranes. The bands obtained from immunoblotting were quantified by scanning densitometry (Sicon, Suffolk, UK).

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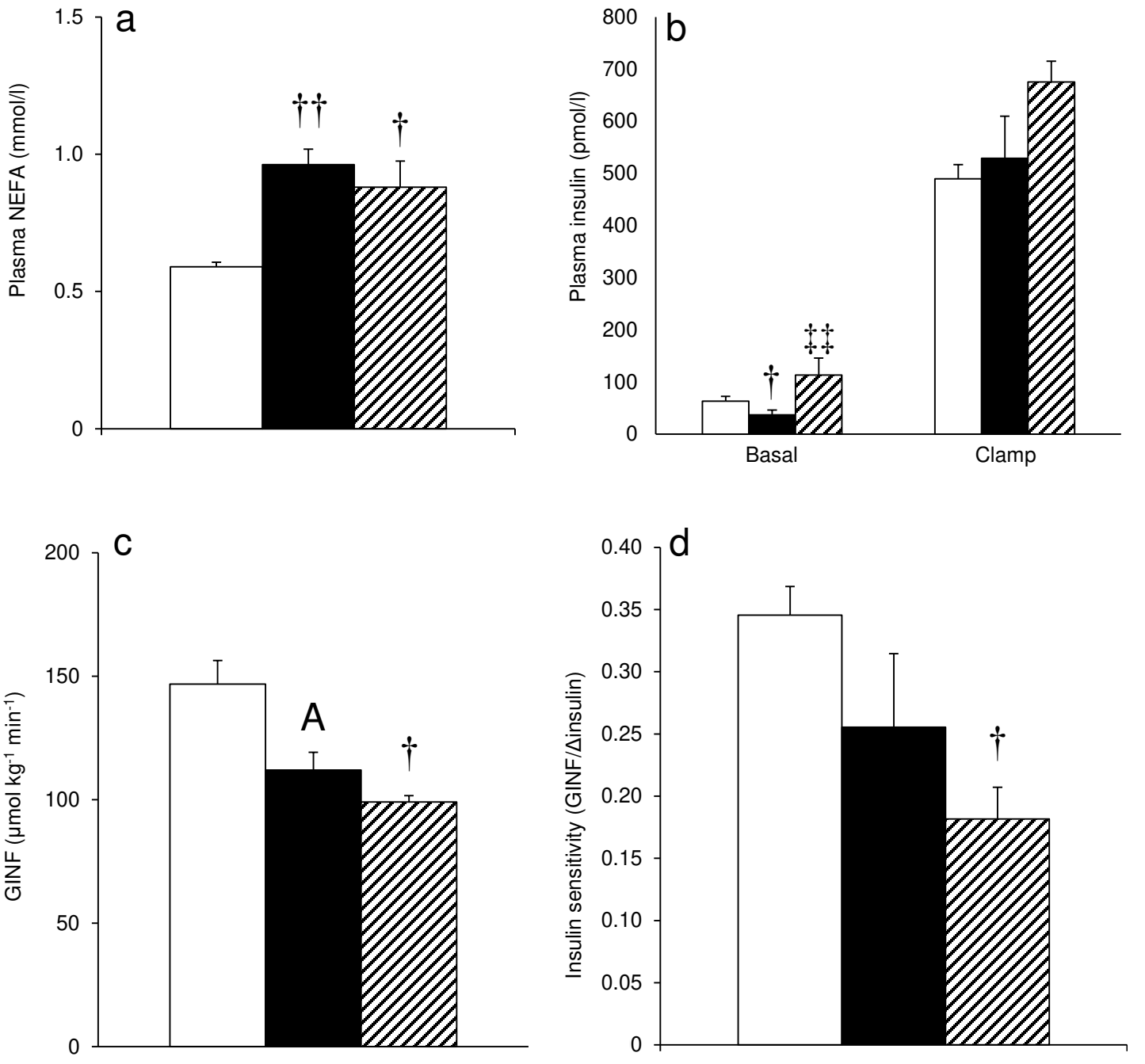
ESM Table 1

Mouse primers for mRNA of proinflammatory genes.

Gene	Catalogue#
Il1b	Rn00580432_m1
Tnfa (*Tnf*)	Rn99999017_m1
Mcp1 (*Ccl2*)	Rn00580555_m1
Il1ra (*Il1rn*)	Rn00573488_m1
Tgfb (*Tgfb1*)	Rn99999016_m1
Cd68	Rn01495634_g1
Cox2 (*Ptgs2*)	Rn01483828_m1

Mouse primers were used for quantitative PCR with commercial TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA).

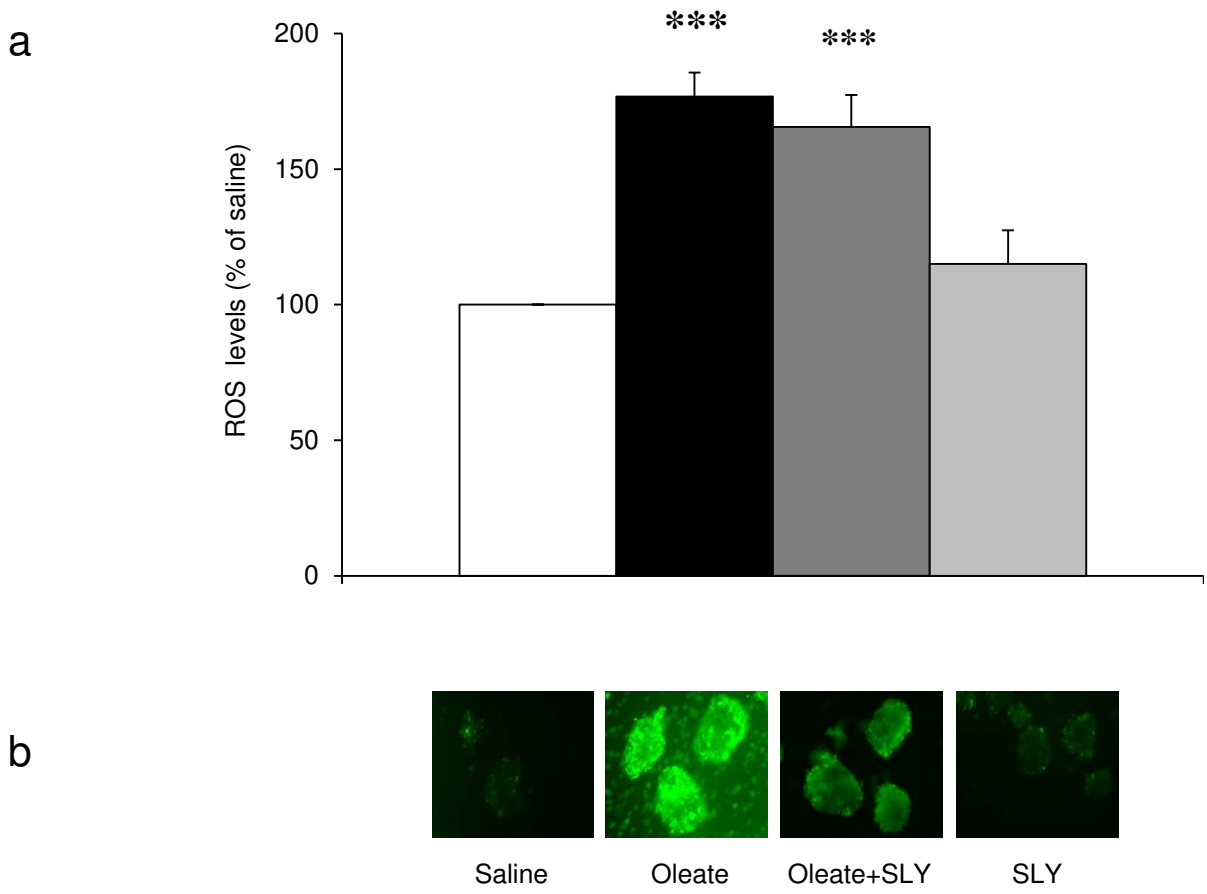
ESM Fig. 1



ESM Fig. 1 Effects of oleate (1.3 µmol/min) and olive oil (20% olive oil infusate containing 50U/ml heparin at 5.5 µl/min) on NEFA (**a**), insulin (**b**), glucose infusion rate (GINF); **c**) and insulin sensitivity (**d**) during hyperinsulinaemic-euglycaemic clamps following 48 h oleate or olive oil infusion. Rats were treated with saline (n=4), oleate (n=4), or OLO (n=3). During 30 min preceding the clamp ('basal period'), measurements were taken at 10-min interval for plasma glucose, insulin and NEFA. At the onset of the clamp, an infusion of human insulin at 5 mU kg^{-1} min^{-1} was initiated and continued for 120 min. To maintain euglycaemia during insulin infusion (basal glucose ~6.5±0.3 mmol/l), a variable infusion of 20% glucose was given through the jugular catheter and adjusted according to glycaemic determinations every 5 min.

Data are mean ± SEM. † $p<0.05$ and †† $p<0.01$ vs. saline; ‡‡ $p<0.01$ vs. oleate
A $p<0.1$ vs. saline
White bar, saline; black bar, oleate; diagonal striped bar, OLO
OLO, olive oil

ESM Fig. 2



ESM Fig. 2 Effects of salicylate on ROS levels (**a**) of freshly isolated islets of fat-infused rats. Rats were treated with saline (n=5), oleate at 1.3 µmol/min (n=6), oleate+salicylate at 0.7 µmol kg^{-1} min^{-1} (n=8), or salicylate alone (n=4). Data are mean ± SEM. *** $p<0.001$ vs saline and SLY. **b**: Representative fluorescent images for ROS (200X).

White bar, saline; black bar, oleate; dark grey bar, oleate + SLY; light grey bar, SLY
SLY, salicylate