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Links between enhanced fatty acid flux, protein kinase C and NFκB activation, and apoB–lipoprotein production in the fructose-fed hamster model of insulin resistance

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ABSTRACT

In the current study, we show evidence, in a fructose-fed hamster model of insulin resistance, that free fatty acid (FFA) can induce hepatic insulin resistance in part via PKC activation leading to increased production of atherogenic apoB100-containing lipoproteins. Interestingly, IκB-kinase β (IKKβ)-dependent NF-κB was activated in hepatocytes from the fructose-fed hamster as an indication for PKC activation. Treatment of hepatocytes with oleate for 16 h showed the activation of the PKC isoforms, PKCα/βII, in a dose dependent manner. Strikingly, the general PKC inhibitor, bisindolylmaleimide-I, Bis-I (5 μM) was found to ameliorate fructose-induced insulin resistance, restoring the phosphorylation status of PKB and suppressing apoB100 overproduction in *ex vivo* and *in vivo*. The data suggest that hepatic PKC activation, induced by increased circulating FFA may be an important factor in the development of insulin resistance and dyslipidemia seen in the fructose-fed hamster model.

FFAs have been implicated as an important causative factor in the development of type 2 diabetes [1]. The FFA-induced impairment of glucose metabolism in muscle is well documented [2–7], but little is known about the mechanisms of the FFA-induced impairment of glucose metabolism and the impairment of the insulin signaling cascade in the liver [8]. In the liver, FFAs increase gluconeogenesis [9], and a large number of studies have demonstrated that intralipid and heparin increase endogenous glucose production during euglycemic clamps [10–14]. Randle et al. [15] have shown that FFA competes with glucose for substrate oxidation.

FFA-induced protein kinase C (PKC) activation has been investigated as a potential mechanism responsible for the FFA-induced insulin resistance in muscle [9]. It has been observed that in hepatocytes, oleic acid promotes translocation of PKC from the cytosol to the plasma membrane [16]. *In vivo* studies conducted in the liver have shown FFA-induced impairment in insulin clearance [17–19]. The mechanism for this impairment is likely a decrease in hepatic insulin binding caused in part by FFA-induced PKC activation. In our current study we attempted to measure the expression level of the Inhibitor κB alpha (IκBα) as a marker for PKC activation and the induction of Nuclear Factor κB pathway (NFκB) in the fructose-fed hamster model. Boden et al. [20] have demonstrated that FFA caused hepatic insulin resistance. FFA was found to induce insulin resistance through activation of proinflammatory NFκB in rat liver [20].

The present study was performed (1) to investigate, whether fructose feeding may turn on PKC and NFκB activation in the hamster model and (2) whether, PKC activation is linked to hepatic apoB100 production. We used the fructose-fed hamster model in order to test the assumption that fructose feeding increases the flux of FFA to the liver and in turn contributes to the development of insulin resistance and metabolic dyslipidemia.

Experimental methods

Materials. Fetal bovine serum, liver perfusion medium, hepatocyte wash medium, liver digest medium, and hepatocyte attachment medium were obtained from Life Technologies (Grand Island, NY). Fatty acid free bovine serum albumin, protein

Abbreviations: FFA, free fatty acid; MAP, mitogen activated protein; PKC, protein kinase C; NFκB, nuclear factor κB.

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A–Sepharose, oleate, and palmitate were from Sigma Chemical Co. (St. Louis, Missouri). PKB, phosphospecific phospho-serine^{473} PKB, and IκBα antibodies were from Upstate Biotechnology (Lake Placid, NY).

Animal protocols and isolation of primary hamster hepatocytes. Male Syrian golden hamsters (*Mesocricetus auratus*) were purchased from Charles River (Montreal, Que.). All animals were placed on either the control diet (normal chow) or a fructose-enriched diet (hamster diet with 60% fructose, Dyets Inc., Bethlehem, PA) for 2 weeks. At the end of the 2-week feeding period, hamsters were fasted overnight and were anesthetized. Primary hepatocytes were digested by digest medium (Life Technologies). Cells were washed three times in hepatocyte wash medium then they were resuspended in serum-free hepatocyte attachment medium (Life Technologies, Inc.) as it transferred into culture medium. After 4 h incubation at 37 °C, 5% CO_2, attached cells were used to carry out the experiments [21].

Chemiluminescent immunoblot analysis. Treated cells were lysed in solubilizing buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% NP-40, 2 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, and 2 mM sodium orthovanadate) and the total cell lysates were subjected to immunoblotting for IRβ, total PKB/Akt, as well as their phosphorylated forms serine 473 and IκBα either directly or after immunoprecipitation against a target protein. For immunoblotting, samples were resolved by SDS–PAGE [22], transferred electrophoretically overnight at 4 °C onto nitrocellulose membranes or PVDF. The membranes were incubated with the antiserum indicated, washed, and then incubated with a secondary antibody. Enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech) was used and membranes were exposed to Kodak Hyperfilm. Films were developed and quantitative analysis was performed using an Imaging Densitometer (FluorChem and Alpha Innotech Corporation).

Proteomic screen and kinetworks TM KPPS-1.1 (phosphatase screen) and KPSS-4.0 (phosphoprotein screen). Primary hepatocytes were extracted, lysed in 0.5 ml of lysis buffer (2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, pH 7.2, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, 5 μM pepstatin A, 10 μM leupeptin, and 0.5% Nonidet P-40. The homogenate was subjected to ultracentrifugation for 30 min at 100,000*g* using a Beckman Table Top TL-100 ultracentrifuge. The resulting supernatant fraction was removed and immediately assayed for its protein concentration. Samples were suspended in Laemmli buffer and these were submitted to Kinexus Bioinformatics Corporation (Vancouver, Canada) for analysis using Kinetworks TM KPKS-1.0 and KPSS-4 (both phosphatases and phosphoprotein) [23]. Any change for any protein expression greater than 25% was considered significant and further investigated by Western blot methodology.

Metabolic labeling of intact primary hamster hepatocytes. Primary hamster hepatocytes were washed with Williams E and Bis-I (5 μM) was added for 16 h then pulsed with [^{35}S] methionine. Media samples and cell lysates were collected after 2 h, subjected to immunoprecipitation, and then analyzed by SDS–PAGE and fluorography [24].

Statistical methods. All results are expressed as means ± SE. Statistical calculations were performed using a 2-tailed Student's *t*-test. A value is considered significant at $p < 0.05$.

Results

Phosphosite screening of primary hepatocytes, comparing control and fructose-fed hamster

We used a proteomics approach to screen candidate molecules for phosphorylation. The phosphosite screen used demonstrates a 45% reduction in the PKB phosphorylation (T308) and a 19% reduction for the alpha (S21) and 33% for beta (S9) glycogen synthase kinase 3 in the fructose-fed hamster in comparison with the control, the chow-fed hamster. However, there was an increase of 112% for the mammalian target of rapamycin phosphorylation (S2448) (Fig. 1A). Although fructose feeding showed a 23% reduction in phosphorylation of the PKC delta isoform, a 22% reduction in mu isoform and 58% reduction for zeta isoform, there was no obvious change in other PKC isoforms as the alpha/beta with only 2% reduction (Fig. 1B). However, the phosphosite screen also showed in-

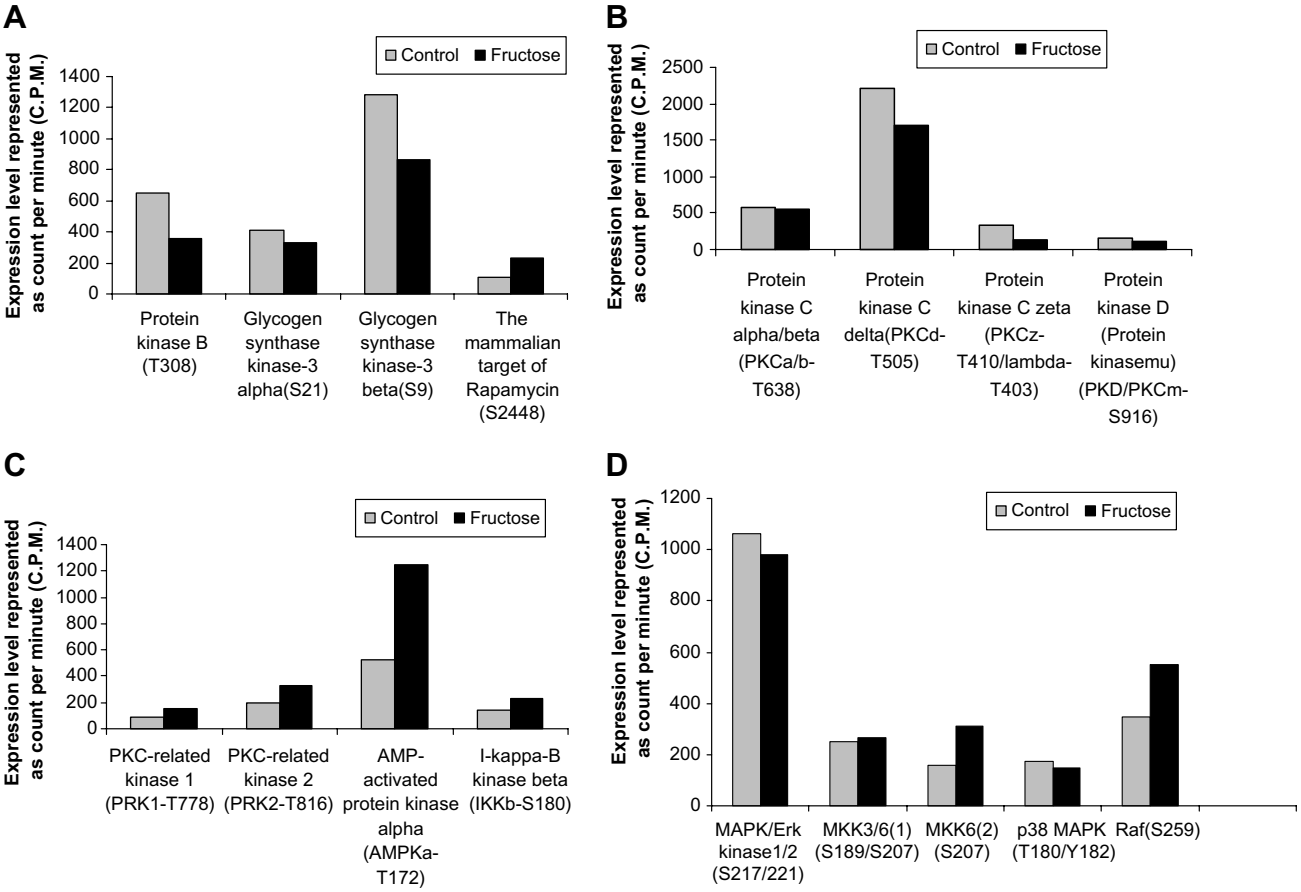

Fig. 1. Phosphosite and phosphatase screening of liver proteins from chow-fed and fructose-fed hamster. Primary hepatocytes from the control (chow-fed) and the fructose-fed hamster were subjected to lysis (see Experimental methods). The homogenate, a pool of three different experiments ($n = 3$), was subjected to ultracentrifugation for 30 min at 100,000*g*. The resulting supernatant was removed and immediately assayed for its protein concentration. Samples were submitted to Kinexus Bioinformatics Corporation (Vancouver, Canada) for Kinetworks. All the screens performed used multi-immunoblotting with prevalidated antibodies.

creased levels in the phosphorylation of PKC-related kinases 1 and 2 (64% and 66%, respectively) when compared between primary hepatocytes from chow and fructose-fed hamsters. There was a 138% increase in AMP-activated protein kinase alpha (T172) as well as a 63% increase in the IκB-kinase β (IKKβ-S180) phosphorylation in the fructose-fed hamster compared with control (Fig. 1C). In Fig. 1D, the phosphosite screen also revealed that fructose feeding induce the activation of the Ras-MAPK pathway, it increased the phosphorylation of Raf by 58%, 96% for both MEkk2 and MEKK6 kinases with reductions of 19% in the MAPK/ERK and 15% for P38 MAPK kinases (Fig. 1D).

Assessment of PKC activation in control and the fructose-fed hamster primary hepatocyte

Fig. 2A shows a 27% reduction in the expression level of IκBα in primary hepatocytes extracted from fructose-fed hamster in comparison with chow-fed controls. Fig. 2B shows an increasing dose–response for the effect of 0–2000 μM oleate on p-PKCα/β activation in primary hepatocytes extracted from chow-fed hamster. There was no change in the albumin expression level that was used as an internal control.

Inhibition of PKC activation enhances PKB phosphorylation in the fructose-fed hamster

In Fig. 3 we used the PKC chemical inhibitor 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (Bis-I) to verify its effect on PKB phosphorylation status. Bis-I was used in both fructose as well as chow-fed hamsters. The results showed that Bis-I was able to reverse fructose-induced insulin resistance and restore the phosphorylation status of PKB and increased the PKB phosphorylation in cells treated with both insulin and Bis-I ($p < 0.001$) in panel A. There was no observable change in PKB mass as seen in panel B.

The link between PKC activation and hepatic apoB100 production in the fructose-fed hamster

Fig. 4A shows the effect of *ex vivo* Bis-I treatment of primary hamster hepatocytes on apoB100 synthesis. In the fructose-fed hamster (FF), there is a significant decrease when the cells are treated with Bis-I for 16 h (26% of untreated control, $p < 0.006$). The effect of the drug was then assessed *in vivo*. Fig. 4B shows the effect of Bis-I (50 μg) infusion *in vivo*. There was a significant decrease in phospho-PKCα/βII (33% of saline control, $p < 0.018$). There was a corresponding significant decrease in apoB100 (32% of saline control, $p < 0.02$) indicating a correlation between the decrease in apoB and phospho-PKCα/βII. Albumin expression was unchanged indicating that this was not a global protein effect, and was specific to apoB100.

Discussion

The deleterious role of FFA in the pathogenesis of type 2 diabetes has been well described though its mechanism is not com-

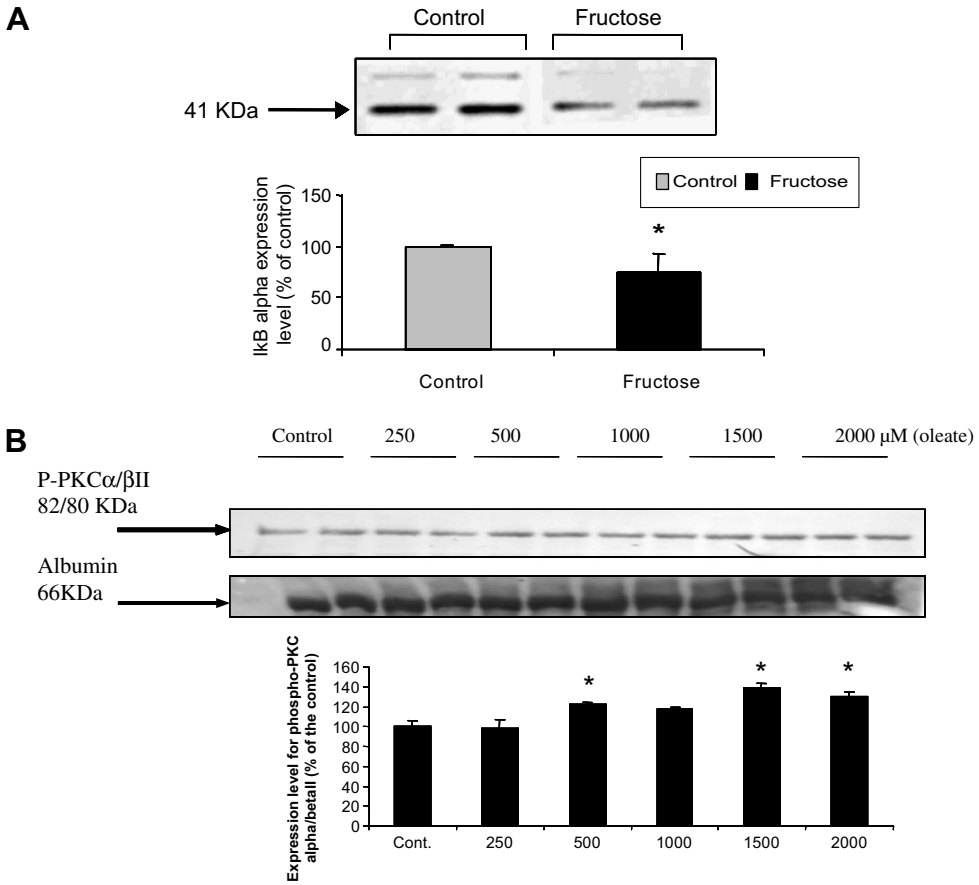

Fig. 2. Assessment of PKC activation in control and fructose-fed hamster primary hepatocytes. Protein expression level for (A) IκBα and (B) effect of oleate on phospho-PKCα/βII activation. Cells were treated with 0–2000 μM oleate for 16 h. Albumin mass was used as a control protein. Each blot is representative of one individual experiment. Statistical analysis for the study was performed using three independent experiments ($n = 3$) each carried out in duplicate, graphs represent means ± SE. Two-tailed *t*-test was conducted comparing the fructose-fed versus the chow-fed hamster data.

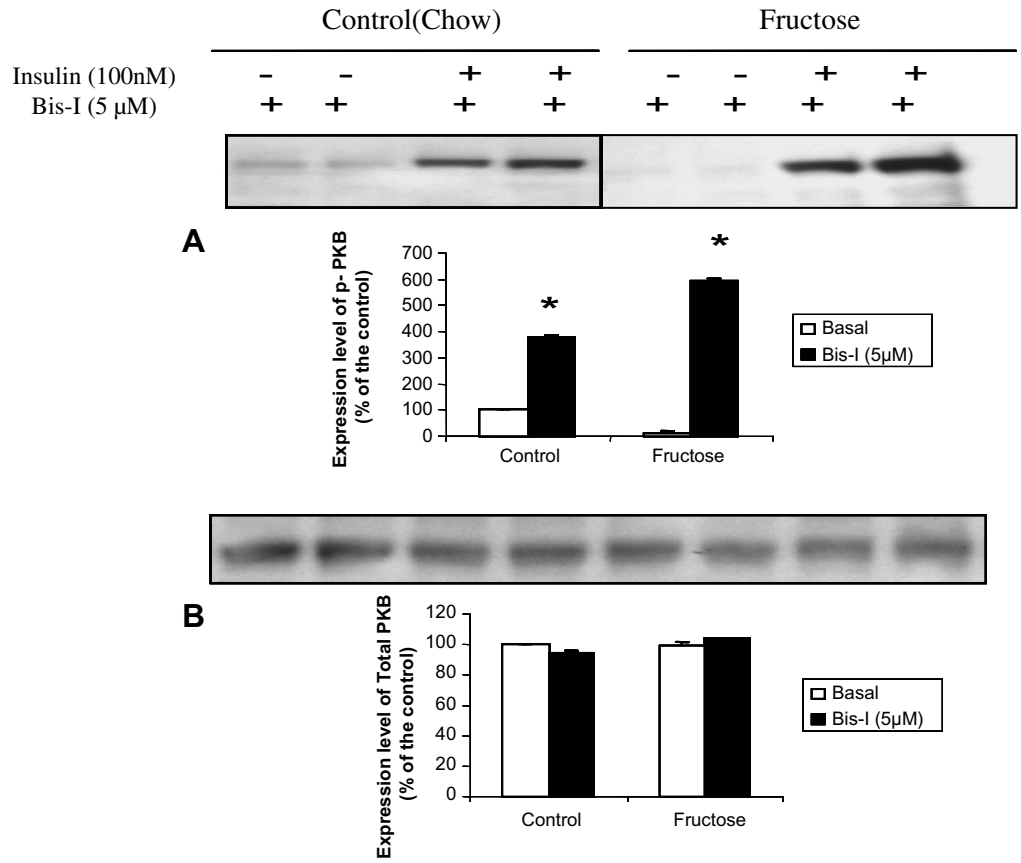

Fig. 3. Effect of inhibition of PKC activation on PKB phosphorylation status Primary hepatocytes from chow or fructose-fed hamsters were treated with Bis-I (5 μM) for 16 h. Immunoblots were performed using total (Fig. 3B) and p-PKB (serine 473) antibodies (Fig. 3A). Each blot is a representation of one individual experiment. Statistical analysis for the study was conducted from three independent experiments ($n = 3$) and each carried out in duplicate, the bar graphs represent means ± SE. Two-tailed *t*-test was conducted comparing fructose-fed versus the chow-fed hamster data.

pletely understood. We employed the fructose-fed hamster as a model of insulin resistance in order to investigate the role of fatty acids in mechanisms of insulin resistance and how this impacts on the dyslipidemia seen in the model [21, 25]. The rationale for the current study is testing the hypothesis that fructose-feeding results in FFA flux to the liver and that excessive fatty acid esterification could be implicated in hepatic insulin resistance that may, in turn, lead to accumulation of DAG, TG and ceramides. Increased FFA flux following fructose feeding may directly activate PKC that indirectly induces perturbations in key signaling molecules of the insulin signaling pathway and its downstream targets of the receptor *in vivo*. Although the skeletal muscle and adipose tissues respond to FFAs directly, the liver increases the glucose output by increasing gluconeogenesis and indirectly affecting insulin signaling [26, 27]. This effect might be explained through direct effect of FFAs on the PKC activation that indirectly affects the insulin signaling and the attenuation of the down stream targets of the PI3K pathway in the liver as well as its effect on *de novo* lipogenesis.

In our model, we intended to investigate the role of PKC involvement in the development of insulin resistance, perturbations in signaling and dyslipidemia as a result of fructose feeding. The main features for the model following the fructose feeding were the increase of plasma levels of cholesterol, triglycerides, free fatty acids, and the insulin level with a normal glucose level in comparison with the chow-fed animal [28]. We asked the question: does fructose feeding increase the flux of FFA to the liver and directly activate PKC and impose its effect indirectly on the PI3K and insulin signaling pathway. To test the current assumption, we used phosphosite proteins [31] and phosphatase screen [27] to search for specific targets involved in the insulin signaling comparing the chow and fructose-fed hamsters to systematically investigate the phosphorylation cascades evoked by fructose feeding and the effect of FFAs in primary hepatic cells. The proteomic data clearly showed a trend of increased levels in the phosphorylation of PKC-related kinases 1 and 2 in the fructose-fed hamster. It also showed a reduction in the expression level for PKC isoforms (PKCα/βII, δ, ζ/γ, and μ) in cells extracted from the fructose-fed hamster, which is an indication of their degradation following prolonged activation *in vivo*. Previous work has clearly demonstrated that prolonged treatment of cells with phorbol esters (PKC activators), often cause reduced levels of PKC isozymes primarily as a result of proteolysis of activated PKC isozymes rather than of diminished synthesis [29].

To confirm the role of PKC activation and its implication in our experimental model, the p-PKCα/βII level was measured following treatment with oleate (0–2000 μM). The results showed a significant increase in a dose dependent manner in agreement with previous report [16]. We also measured the protein expression level for IκBα, it showed a 27% reduction in the expression level of IκBα as well as a 63% increase in the IκB-kinase β (IKKβ-S180) phosphorylation in primary hepatocytes extracted from fructose-fed hamster in comparison with chow-fed controls. Moreover, we assessed the PKC inhibition and its impact upon PKB as a downstream target for PI3K pathway activation following insulin stimulation in the fructose-fed model. The results demonstrate the impact of PKC activation seen in this model as a result of FFA flux. Additional supportive evidence is the amelioration of the PKB phosphorylation status (serine^{473}) in primary hepatocytes ex-

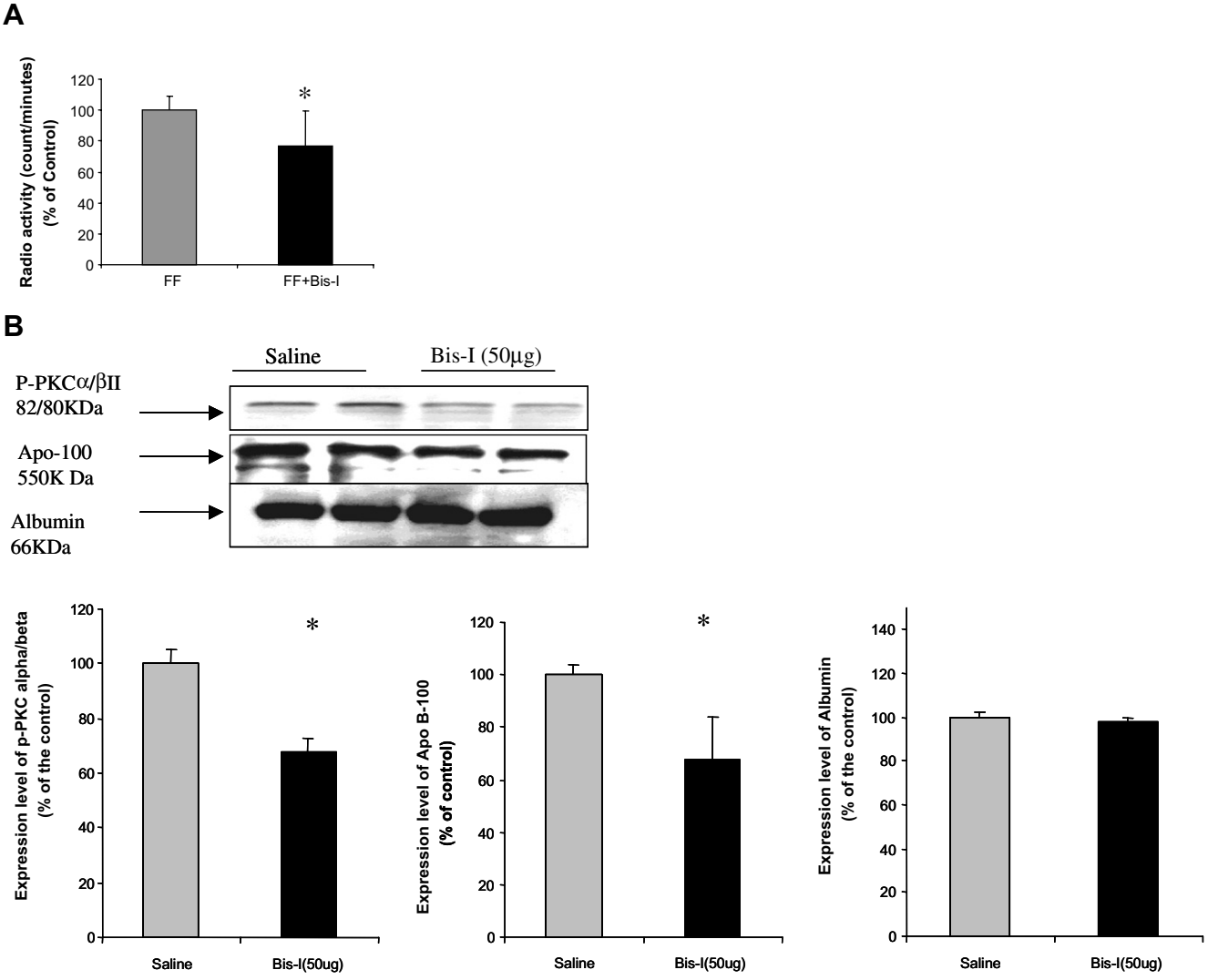

Fig. 4. The link between PKC activation and apoB100 synthesis as a marker for dyslipidemia in the fructose-fed hamster. (A) Effect of 16 h Bis-I (5 μM) treatment on primary hepatocytes from fructose-fed hamster (FF) on apoB100 synthesis. Hepatocytes were washed with Williams E, Bis-I (5 μM) was added for 16 h then pulsed with [^{35}S] methionine. Media samples and cell lysates were collected after 2 h, subjected to immunoprecipitation, and then analyzed by SDS–PAGE and fluorography. (B) Chow-fed hamsters were infused with either Bis-I (50 μg) or saline. Phospho-PKC α/βII, apoB100 synthesis/mass, and albumin mass were measured by immunoprecipitation and immunoblotting.

tracted from the fructose-fed hamster in comparison to control cells extracted from the chow-fed animals. Our findings clearly demonstrate that the inhibition of PKC following Bis-I treatment in the fructose-fed hamster was able to reverse insulin resistance and PKB inhibition seen in the fructose-fed hamster [30]. It clearly enhanced both insulin sensitivity and PKB phosphorylation.

We also attempted to test and investigate the effect of PKC activation and its impact on apoB100 in our fructose-fed hamster model. We determined whether fructose feeding induces activation of PKC and affect apoB100 synthesis and VLDL production. Previous studies from our laboratory have demonstrated that hepatic VLDL–apoB100 overproduction is associated with attenuated hepatic insulin signaling [30]. Our results demonstrate that Bis-I suppresses the increased synthesis of apoB100 in the fructose-fed model. To further confirm our finding, we have conducted an *in vivo* experiment. The results show that Bis-I infusion reduced the expression level for the phospho-PKC α/βII and production of apoB100. This demonstrates the importance of PKC activation in the fructose-induced VLDL production observed in the hamster model. PKC activation as a mechanism might occur as a result of FFA flux to the liver following 2 weeks of fructose feeding which results in insulin insensitivity and dyslipidemia through the stabilization and overproduction of apoB100. It has been shown that the release of ceramide after membrane sphingomyelin hydrolysis decreases the basolateral secretion of triacylglycerol and apoB in cultured human intestinal cells through the inhibition of PKC [31].

In conclusion, we investigated potential mechanisms for FFA-induced hepatic insulin resistance and dyslipidemia in the fructose-fed hamster model. We demonstrated that increased fatty acid influx to the liver and the resulting PKC activation in the fructose-fed model are important events that contribute to hepatic apoB100 overproduction commonly observed in insulin resistant states. In addition, our current data point to FFA-induced PKC activation and its impact on dyslipidemia as a potential therapeutic target in the treatment of diabetes and insulin resistance.

Conflict of interest

This work was supported by the Hospital for Sick Children in affiliation with the University of Toronto. Rafik Ragheb is a recipi-

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