Original Article

The effects of dietary obesity on protein expressions of insulin signaling pathway in rat aorta

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ABSTRACT: The deleterious effects of obesity on insulin response in vasculature may be due to changes in various components of insulin signaling pathway. Therefore, this study was designed to investigate effects of dietary-obesity, removal of palatable diet, and fenofibrate treatment on protein expressions of insulin signaling pathway in rat aorta. Adult male Wistar rats were fed either standard chow or a palatable diet (untreated obese animals) for 15 weeks. Another group of rats were fed the palatable diet for 8 weeks followed by standard chow for further 7 weeks, while a further group were fed the palatable diet for15 weeks and were dosed with fenofibrate (50 mg/kg/day) for the last 7 weeks. Untreated obese animals had significantly higher body weight than other three groups ($p < 0.05$ for all). There were no significant differences between IR-β, IRS1 and IRS2, Akt, Shc, and ERK1/2 levels in chow-fed and untreated obese animals, while PI 3-kinase level were significantly ($p < 0.0001$) decreased in untreated obese animals. Chronic removal of palatable diet completely reversed the levels of PI 3-kinase to the normal while, fenofibrate treatment further reduced PI 3-kinase levels. On the other hand, there was a significant ($p < 0.05$) increase in eNOS in untreated obese animals compared with chow-fed controls. This effect was reversed by removal of palatable diet and fenofibrate treatment. These data suggest that dietary-obesity selectively inhibits PI 3-kinase while, removal of obesity-inducing diet improves PI 3-kinase levels which may have a role in vascular reactivity.

Keywords: Obesity, Insulin resistance, Insulin signaling pathway, Vascular function, PI 3-kinase, Fenofibrate

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1. Introduction

Obesity, characterized by excess adipose tissue is now becoming a worldwide epidemic (*1,2*). Various studies have suggested that obesity *per se* is an independent cardiovascular risk factor (*3*), as well as predisposing to type 2 diabetes, hypertension and dyslipidaemia (*4*). Furthermore, obesity induces insulin resistance which is associated with development of cardiovascular diseases that include hypertension (*5*), and reduced endothelial function (*6*). Insulin has a protective role in vascular function. It stimulates nitric oxide (NO) production, leading to vasorelaxation. Insulin-induced NO-dependent vasorelaxation is markedly decreased in obesity (*7*), however, the mechanism(s) of decrease in insulin-induced vasorelaxation is not fully understood. Insulin has other major physiological roles that include facilitation and increase of amino acid transport, glycogen synthesis, DNA synthesis and gene expression (*8*). Moreover, it specifically enhances release of nitric oxide (*9,10*), regulates mRNA matrix proteins (*11*) and constitutive endothelial NO synthase (*12*) activity in vasculature.

In vascular cells, the effects of insulin are initiated through binding to the insulin receptor alpha subunit (IR-α), which activates the intrinsic receptor tyrosine kinase (*13*), resulting in autophosphorylation of insulin receptor beta subunit (IR-β) and tyrosine phosphorylation of intracellular adaptor proteins - insulin receptor substrates (IRS-1 and IRS-2) (*14*) and Shc (*15*). Tyrosine phosphorylated IRS-1 or IRS-2 then binds to src-homology 2 (SH2) domains of intracellular proteins, including the p85 regulatory subunit of phosphatidyl-inositol (PI) 3-kinase (*16*). The interactions of IRS and p85 subunit of PI 3-kinase results in the activation of p110 catalytic subunit of PI 3-kinase. Activation of PI 3-kinase increases serine phosphorylation of Akt which in turn, directly phosphorylates eNOS on serine 1177 and activates the enzyme, leading to increased NO production and thus providing vascular protection (*17*). Tyrosine

phosphorylated Shc and IRS proteins can also bind to SH2 domain of Growth factor Receptor-protein Bound 2 (GRB2), leading to the activation of the Ras-Raf-MAP kinase signal pathway that is associated with gene expression and cell growth (*18*). PI 3-kinase expression and activation has been linked to NO production, whereas, Raf-MAP kinase pathway is associated with the growth of vascular cells and the expression of extracellular matrix proteins (*10*). Furthermore, various studies (*7-12*) have shown attenuation of insulin-induced vasorelaxation in obesity, however, there is little information on the mechanism(s) of diet-induced changes in insulin signaling pathway in vasculature. Therefore, it is possible that changes in any component of insulin signaling pathway may alter vasorelaxant property of insulin. Furthermore, the level of protein expression of these kinases could fluctuate and respond differently to any pathological, physiological, or pharmacological conditions, such as dietary obesity and its treatment. Therefore, investigating the protein expression of these kinases under certain obesity-related experimental conditions could provide vital information about obesity-induced insulin resistance in vascular tissues. Consequently, we aimed in this study to investigate the protein expression of insulin signaling components in aorta of four different experimental groups, namely, 1) control chow-fed lean animals, 2) untreated diet-induced obese animals, 3) obese animals following chronic withdrawn of palatable diet, and 4) obese animals treated with fenofibrate. Fenofibrate has been shown to improve lipid profiles, reduce adiposity, and may have a role in correction of vascular function in obesity.

2. Materials and Methods

2.1. *Animal and experimental protocol*

All procedures used in this study were approved by the institutional committee and accord with current UK legislations. Adult male Wistar rats (n = 28, 190 ± 3 g) were randomized and assigned to a control group (n = 7) and a test group (n = 21). All animals had free access to water and were housed individually under controlled environmental conditions (19-22°C; 30-40% humidity) and a 12 h light-dark cycle (lights on at 07:00 h). Controls were fed standard laboratory pelleted diet (Chow-fed; CRM Biosure, Cambridge, UK), while test group had free access to a highly-palatable diet consisting of (by weight) 33% ground pellet diet, 33% Nestlë condensed milk, 7% sucrose and 27% water. The energy content of chow was: 60% as carbohydrate, 30% as protein, and 10% as fat, and that of palatable diet was 65% as carbohydrate, 19% as protein and 16% as fat. Chow-fed controls remained on their prospective diet for 15 weeks, while after 8 weeks palatable-diet-fed animals were subdivided into three subgroups (each group 7 animals). In the first subgroup, palatable diet was removed and the standard chow diet was re-introduced (diet-to-chow), while the second subgroup remind on palatable diet and were given fenofibrate (fenofibrate-treated, 50 mg/kg/day) by oral gavage for further 7 weeks and the third subgroup (diet-fed) was given vehicle (1% carboxymethyl cellulose at 1 mL/kg body weight; Sigma, UK), by oral gavage daily for 7 weeks. On the day of experiment (after 15 weeks), the rats were killed by CO_2 inhalation after 2 h of fasting and the aorta was dissected and snap frozen in liquid nitrogen for molecular studies.

2.2. *Protein extraction*

Fifty milligram of aorta was homogenized at 4°C in 500 µL buffer containing 120 mM NaCl, 10% glycerol, 2 mM Na_3VO_4, 1% Nonidet-P40, 1 mM PMSF, 10 mM $Na_4P_2O_7$, 100 mM NaF, 20 mM Tris (pH 7.6) and a complete mini® protease inhibitor cocktail with polytron homogenizer. The homogenates were then incubated on a rocking platform at 4°C for 30 min. After 3 × 10 sec bursts of sonication, tubes were subsequently centrifuged for 45 min at 13,000 × g at 4°C. Supernatants were collected, and protein concentrations were determined by the BCA method kit.

2.3. *Western-immunoblotting*

2.3.1. *SDS-PAGE*

A discontinuous acrylamide gel system was used. A stacking gel (5%) was set above a 10% (depending on protein of interest) resolving gel. Samples were standardized to 4 mg/mL with lysis buffer. 20 µL of sample protein was boiled in 20 µL 2 × electrophoresis sample buffer, for 10 min and then subjected to SDS-PAGE (Tris-glycine buffer, 100 V). Resolved proteins were electro blotted onto nitrocellulose membranes in buffer containing 25 mM Tris, 190 mM glycine, 1% SDS (w/v) and 20% (v/v) methanol (100 V for 1 h). Staining immobilized proteins on nitrocellulose with Ponceau S assessed successful transfer. Nitrocellulose blots were sub-merged in 0.2% Ponseau S for 15 min with agitation. Blots were then washed with 1 × PBS with 1% Tween, until proteins could be visualized. Blots were then washed until protein bands had disappeared.

2.3.2. *Immunoblotting*

Non-specific binding proteins were prevented by incubating the blot with blocking buffer (5% milk powder, 1× PBS) at room temperature for one hour, followed by immunoblotting with appropriate primary antibody (1:500 dilution) made up in blocking buffer, left overnight at 4°C. Blots were then washed in 1 × PBS with 1% Tween and incubated with secondary antibody (1:1,000), a HRP linked anti-rabbit for 1 h at room temperature. Proteins were detected using enhanced

chemiluminescence method. Positive controls were included for standardization of samples between blots and molecular weights markers were used for sizing bands. Densitometry was used to quantify protein bands.

2.4. *Chemicals and antibodies*

SDS-PAGE and immunoblotting equipments were obtained from Bio-Rad (Richmond, Calif., USA). Tris, phenylmethylsulfonylfluoride (PMSF), aprotinin, dithiotheitol, Tween-20 and glycerol were obtained from Sigma Chemicals (Sigma Ltd., UK). Complete mini® protease inhibitor cocktail was obtained from Roche Diagnostics Ltd. (East Sussex, UK). BCA (Bicinconinic acid) method kit for protein determination was obtained from (Sigma Ltd., UK). Secondary antibody, an HRP-linked anti-rabbit was purchased from (Serotic, Oxford, UK). Molecular weight marker was obtained from (BioRad Laboratoties Ltd., Hertfordshire, UK) Polyclonal antibodies against beta subunit of insulin receptor (C-19, sc-711), IRS-1 (C-20, sc-559), IRS-2 (A-19, sc-1556), eNOS (NOS3, C-20, sc-654), Akt1 (C-20, sc-1618), Shc (C-20, sc-288: specific for Shc p46, p52 and p66) and ERK1 (C-16, sc-93: reactive with ERK1 p44 and, to lesser extent, ERK2 p42) and PI 3-kinase were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA).

2.5. *Data interpretation and statistical analysis*

Changes in body weights of each group were collected weekly and are expressed as absolute total body weight respectively. For Western blotting, the data from chow-fed (control) animals were expressed as 100% response, and the results from other three groups were normalized and subsequently expressed as the percentage of their respective controls. Data are expressed as mean ± S.E.M. Data have normal distribution (Shapiro Wilk W test). Statistical significance was tested using Student '*t*-test' or repeated-measures (ANOVA; Bonferroni *t*-test) or the Mann-Whitney test, as appropriate. Results were considered statistically significant at the $p < 0.05$ levels.

3. Results

3.1. *Changes in total body weight*

Animals given palatable diet progressively gained more weight than their chow-fed counterparts. A significant difference in total body weight was observed after 5th week of feeding and further increased after 8th week where diet-fed animals had significantly (> 10%, $p = 0.0041$) higher total body weight than chow-fed controls. At the end of the experiment, body weight of diet-to-chow and fenofibrate treated animals were significantly ($p < 0.01$) lower than untreated diet-fed animals (Figure 1).

3.2. *Protein expression studies*

3.2.1. *IR-β*

There were no significant differences in IR-β protein levels in aorta from chow-fed, untreated diet-fed and diet-to-chow animals, while aorta from fenofibrate-treated animals had significantly (up to 30%, $p < 0.001$) higher IR-β protein levels than chow-fed group (Figure 2).

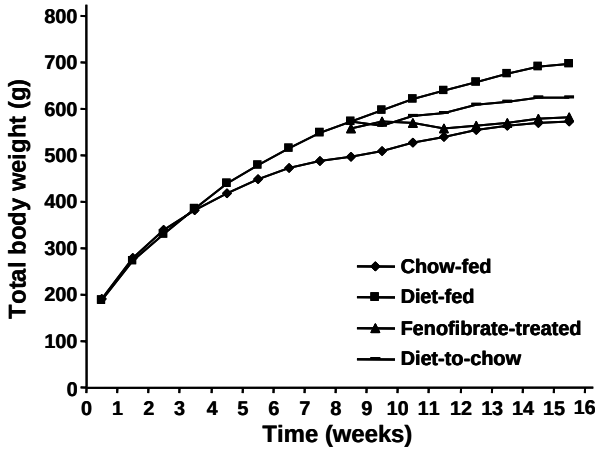

Figure 1. The effect of palatable diet, its removal, and fenofibrate treatment on total body weight in the rat. Animals were fed either standard chow (chow-fed) or a palatable diet (diet-fed) for 15 weeks. Fenofibrate treated group was fed palatable diet for 15 weeks and received fenofibrate (50 mg/Kg/day) for the last 7 weeks, while diet-to-chow group was fed palatable diet for the first 8 weeks and chow for the last 7 weeks of the experiment. At the end of the experiment (15 weeks) diet-fed animals weighed significantly greater than the chow-fed, fenofibrate-treated, and diet-to-chow groups ($p < 0.0002$, 0.002, and 0.02, respectively). The body weight of diet-to-chow group was significantly greater ($p < 0.02$) than the chow-fed group, while that of the fenofibrate-treated group was not significantly ($p < 0.66$) different from the chow-fed group. Data are expressed as mean ± S.E.M.

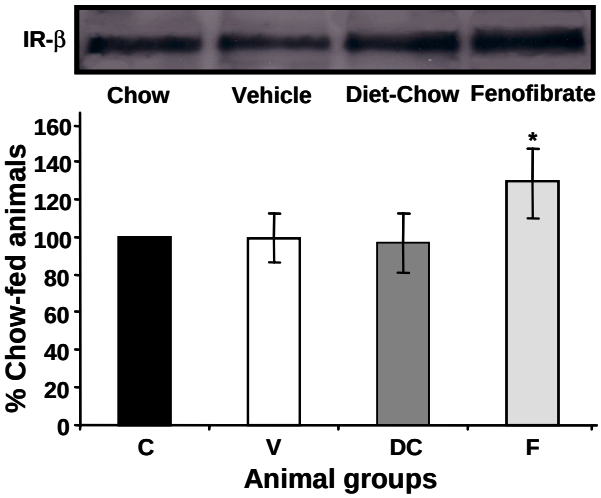

Figure 2. Protein expression of IR-β in the rat aorta. Equal amounts (40 µg/well) of protein were separated by SDS-PAGE and immunoblotted with IR-β antibody. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± SEM; $n = 7$) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * $p < 0.05$ *vs* chow fed.

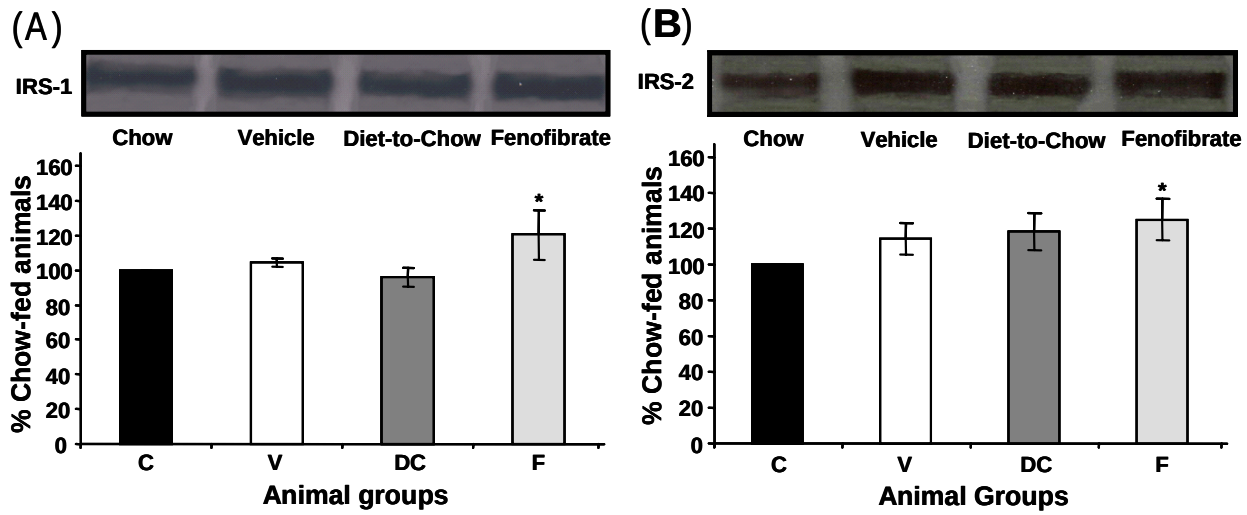

Figure 3. Protein expression of IRS-1 and IRS-2 in the rat aorta. Equal amounts (40 µg/well) of protein were separated by SDS-PAGE and immunoblotted with IRS-1 (A) or IRS-2 (B) antibody. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± SEM; $n = 7$) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * $p < 0.05$ *vs* chow fed.

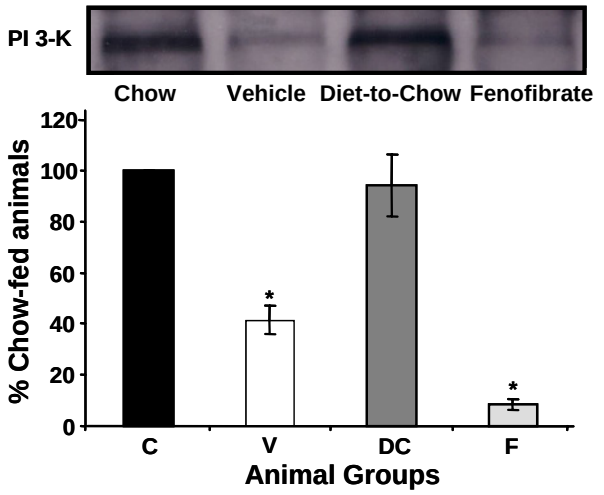

Figure 4. Protein expression of PI 3-K in the rat aorta. Equal amounts (40 µg/well) of protein were separated by SDS-PAGE and immunoblotted with PI 3-kinase antibody. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± SEM; $n = 7$) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * $p < 0.0001$ *vs* chow fed.

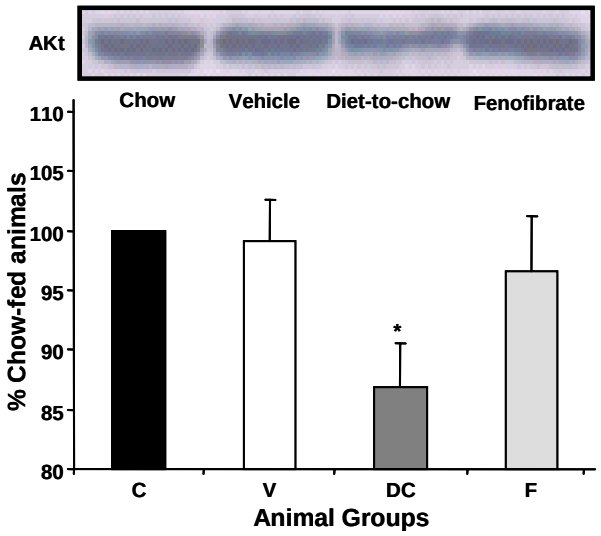

Figure 5. Protein expression of Akt in the rat aorta. Equal amounts (40 µg/well) of protein were separated by SDS-PAGE and immunoblotted with Akt antibody. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± SEM; $n = 7$) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * $p < 0.05$ *vs* chow fed.

3.2.2. *IRS-1 and IRS-2*

As with IR-β, the IRS-1 and IRS-2 levels in chow-fed, untreated diet-fed and diet-to-chow fed groups were comparable, while aorta from fenofibrate-treated animals had significantly higher levels of both IRS-1 (26%, $p < 0.05$) and IRS-2 (25%, $p < 0.05$) compared to chow-fed group (Figure 3).

3.2.3. *PI 3-kinase*

Compared with chow-fed animals, there was a marked reduction in PI 3-kinase levels in untreated diet-fed animals (59%, $p < 0.001$). PI 3-kinase levels were further reduced by fenofibrate-treatment (92%, $p < 0.0001$), while removal of palatable diet completely reversed the reduction in PI 3-kinase levels seen in diet-fed group (Figure 4).

3.2.4. *Akt*

The protein levels of Akt were similar in chow-fed, untreated diet-fed and fenofibrate-treated animals, while aorta from diet-to-chow group had significantly lower (13%, $p < 0.01$) levels of Akt protein (Figure 5).

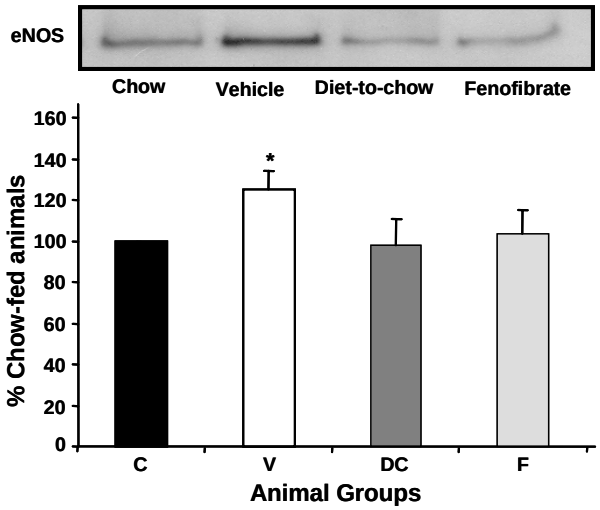

Figure 6. Protein expression of eNOS in the rat aorta. Equal amounts (40 µg/well) of protein were separated by SDS-PAGE and immunoblotted with eNOS antibody. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± SEM; $n = 7$) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * $p < 0.05$ *vs* chow fed.

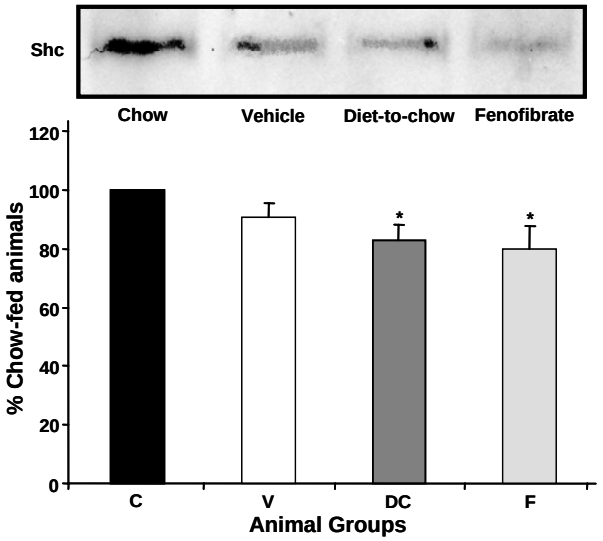

Figure 7. Protein expression of Shc in the rat aorta. Equal amounts (40 µg/well) of protein were separated by SDS-PAGE and immunoblotted with Shc antibody. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± SEM; $n = 7$) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats. * $p < 0.05$ *vs* chow fed.

3.2.5. *eNOS*

There was a significant (25%, $p < 0.001$) elevation of eNOS levels in aorta from untreated diet-fed animals compared with that of chow-fed control, while removal of diet or fenofibrate-treatment markedly reduced the elevation of eNOS protein concentration seen in untreated diet-fed animals (Figure 6).

3.2.6. *Shc*

Although, Shc protein levels were not significantly altered by diet feeding but removal of the diet or fenofibrate-treatment significantly (by up to 20%, $p < 0.05$) attenuated Shc levels in comparison to that of chow-fed controls (Figure 7).

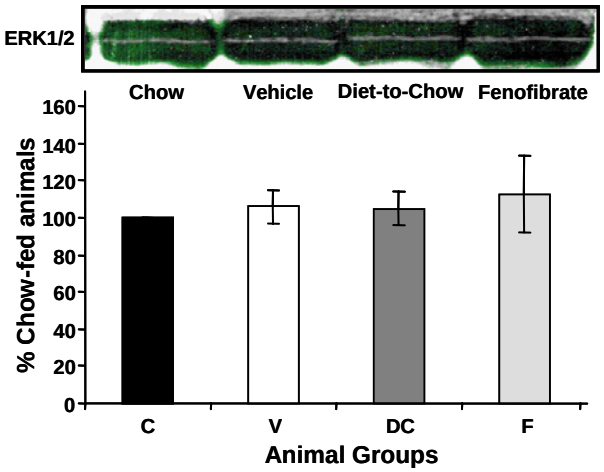

Figure 8. Protein expression of ERK1/2 in the rat aorta. Equal amounts (40 µg/well) of protein were separated by SDS-PAGE and immunoblotted with PERK1/2 antibody. The animal groups are: C) chow-fed lean control, V) Vehicle group (untreated dietary obese), DC) Diet-to-Chow group, and F) fenofibrate treated group. Data (mean ± SEM; $n = 7$) are normalised to that of control group and expressed as a percentage of the chow fed (control) rats.

3.2.7. *ERK1/2*

Immunoblotting analysis of ERK1/2 (MAP kinase pathway) protein in all four groups was remarkably similar to each other. Palatable-diet feeding in the presence or absence of fenofibrate had no effect on ERK1/2 levels nor did the removal of the palatable diet altered ERK1/2 levels in aorta (Figure 8).

4. Discussion

Interactions between insulin, IR-β, IRS-1, and IRS-2 activates PI 3-kinase pathway that results in vasorelaxation. Therefore, the integrity of protein levels and activation of the cascade in insulin signaling pathway in vasculature is a crucial factor in mediating normal vascular functions. In the present study, dietary-obesity did not adversely alter the concentrations of IR-β, IRS-1, and IRS-2, suggesting that reduced vasorelaxation to insulin seen in dietary-obesity (*7,19*) may not be due to the changes in protein levels of IR-β, IRS-1, and IRS-2. This raises possibility that attenuated insulin responses seen in obesity (*7,19*) may be due to changes in protein levels beyond membrane receptors. In fact, the present study indicates a marked decrease in PI 3-kinase levels in aorta from dietary-obese rats, suggesting that dietary-obesity adversely affects PI3-kinase protein level and thereby reducing insulin-induced vasorelaxation. Studies on genetically obese animals have reported similar observation. Jiang and colleagues have shown reductions in IRS-1, and IRS-2 but not IRS-β protein levels in obese Zucker rats in comparison with lean animals (*20*). Moreover, the

same study reported a marked inhibition of PI 3-kinase activation in aorta of fatty-Zucker rats. Although, in this study we did not measure insulin-induced phosphorylation of signaling components, however, low level of PI 3-kinase seen in our study together with reduced functionality of PI 3-kinase reported on Zucker rats (*20*) may play an important role in integrity of endothelial function. A similar finding has also been reported in human umbilical vein endothelial cells where inhibition of PI 3-kinase markedly attenuated insulin-stimulated NO production (*21*), further arguing for a significant role of PI 3-kinase-changes in endothelial dependent insulin-induced vasorelaxation.

We have reported that, in obese animals, removal of obesity-inducing diet completely restores endothelial function (*22*) suggesting that reversal of adiposity might be of benefit in correcting obesity-induced attenuation of PI 3-kinase. In fact, in our present study, PI 3-kinase levels were resorted in diet-to-chow group, further strengthening the hypothesis that PI 3-kinase levels may determine the magnitude of endothelial function in obesity. Therefore, it is possible that any deficiency in the concentrations of PI 3-kinase may participate in inducing insulin resistance in vascular system accompanied with reduced NO production, leading to endothelial dysfunction.

In this study, fenofibrate-treatment further reduced PI3-kinase. Peroxisome proliferator-activated receptor (α) (PPAR-α) plays a crucial role in the control of mitochondrial B-oxidation of fatty acids (*23,24*). Moreover, PPAR-α expressed in the vascular tissue, mainly the endothelial cells (*25*). Therefore, it is plausible to assume that the increased intracellular fatty acids and the presence of abundant amount of PPAR-α agonist (fenofibrate) may enhance fatty acid oxidation leading to increase in the intracellular fatty acid metabolites. Fatty acid metabolites such as fatty acyl-CoA, diacylglycerols, and ceramides shown to alter insulin signaling and induce insulin resistance (*26*) thus causing reduction in PI3 kinase levels in these animals.

Functionally active PI 3-kinase stimulates Akt, which in turn activates eNOS, leading to production of NO and subsequent vasorelaxation (*21*). In our study, protein levels of Akt were similar between dietary-obese and lean aorta while removal of palatable diet caused marked decrease in Akt levels. On the other hand, to our surprise there was a significant increase of eNOS levels in the obese group, whereas, removal of palatable diet or fenofibrate treatment restored eNOS levels to that of lean control group. Although, increased eNOS level in this study is suggestive of augmented endothelial-dependent vasorelaxation, but numerous human and animal studies have shown attenuated endothelial function in obesity (*6,19,27*). There are several possibilities on increased level of eNOS in obese animals. One possible explanation, is activation of a compensatory mechanism to overcome the decrease in NO production or increase inactivation of NO seen in arteries of dietary obese rats as a result of increased oxidative stress (*28*). Moreover, in obese animals there are elevation of insulin concentration and endothelial dysfunction (*22*). Furthermore, insulin is a potent enhancer of reactive oxygen species (ROS) synthesis in endothelial and vascular smooth muscle cells, and superoxide is known to reduce NO and subsequently increased vasoconstriction (*29,30*), thus, the elevation of eNOS could compensate this reduction. Insulin is also shown to induce eNOS expression in endothelial cells (*31,32*) and therefore, insulinaemia which observed in obese rats could directly be responsible for the enhancement of eNOS expression. Similar results and hypothesis have also been reported by others indicating an increase in eNOS levels in Zucker obese coronary (*28*) and cerebral (*33*) arteries. However, the function of eNOS maybe regulated by the PI 3-kinase or MAP-kinase pathway (*34*), and thus decrease in PI 3-kinase seen in untreated obese rats may cancel any beneficial effects of increased eNOS. Furthermore, activated VEGF stimulates eNOS expression at both mRNA and protein levels in a does-dependent manner (*34*), suggesting that the elevated levels of eNOS in dietary-obese group seen in the present study maybe due to increased level of VEGF through MAP-kinase pathway, correlating positively with the dietary-obese subjects (*35*). Therefore, restoration (*i.e*. reduction) of total body fat seen in diet-to-chow and fenofibrate-treated animals, may have resulted in reduced effect of VEGF on eNOS expression (*35*) and thus improved vascular function (*22,36*). Inhibition of PI 3-kinase pathway enhances the mitogenic actions of insulin through MAP kinase pathway (*37*). Hence, it is possible that inhibition of PI 3-kinase pathway seen in both untreated dietary-obese and fenofibrate treated groups may cause over activation of other signaling pathway such as MAP kinase, resulting in the genesis of atherosclerosis and cardiovascular disease.

In conclusion, the present study demonstrates for the first time that long term feeding (15 weeks) of animals with an obesity-inducing palatable diet causes selective changes in protein levels of PI 3-kinase-dependent signaling pathway in aorta. Chronic withdrawal of obesity-inducing diet causes a complete normalization of PI 3-kinase, while fenofibrate treatment failed to improve PI 3-kinase concentrations in dietary-obese animals. A reduction in PI 3-kinase levels may have a role in inducing insulin resistance in vasculature, contributing to increased incidence of cardiovascular events seen in obese subjects. Furthermore, we also postulate that, the inhibition of PI 3-kinase pathway may result in increased activation of MAP kinase pathway leading to an increased proliferation and migration of endothelial cells, thereby increasing the risk of cardiovascular events. However, this hypothesis merits further investigation. Furthermore, the adverse

effects of dietary obesity on insulin transduction in vasculature are post receptor, on the level of PI3-kinase and downstream, and reversible, mainly by removal of obesity-inducing diet, which may have a role in combating diet-induced obesity-related cardiovascular dysfunction.

Acknowledgments

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