Clinical Science (2015) **129**, 1025–1036 doi: 10.1042/CS20150143

Original Paper

Inflammation-induced microvascular insulin resistance is an early event in diet-induced obesity

Lina Zhao*, Zhuo Fu*, Jing Wu*†, Kevin W. Aylor*, Eugene J. Barrett*, Wenhong Cao‡ and Zhenqi Liu*

*Division of Endocrinology and Metabolism, Department of Medicine, University of Virginia Health System, Charlottesville, VA 22903, U.S.A.
†Department of Endocrinology, Central South University Xiangya Hospital, Changsha, Hunan 410008, China
‡Department of Nutrition, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, NC 27516, U.S.A.

Abstract

Endothelial dysfunction and vascular insulin resistance usually coexist and chronic inflammation engenders both. In the present study, we investigate the temporal relationship between vascular insulin resistance and metabolic insulin resistance. We assessed insulin responses in all arterial segments, including aorta, distal saphenous artery and the microvasculature, as well as the metabolic insulin responses in muscle in rats fed on a high-fat diet (HFD) for various durations ranging from 3 days to 4 weeks with or without sodium salicylate treatment. Compared with controls, HFD feeding significantly blunted insulin-mediated Akt (protein kinase B) and eNOS [endothelial nitric oxide (NO) synthase] phosphorylation in aorta in 1 week, blunted vasodilatory response in small resistance vessel in 4 weeks and microvascular recruitment in as early as 3 days. Insulin-stimulated whole body glucose disposal did not begin to progressively decrease until after 1 week. Salicylate treatment fully inhibited vascular inflammation, prevented microvascular insulin resistance and significantly improved muscle metabolic responses to insulin. We conclude that microvascular insulin resistance is an early event in diet-induced obesity and insulin resistance and inflammation plays an essential role in this process. Our data suggest microvascular insulin resistance contributes to the development of metabolic insulin resistance in muscle and muscle microvasculature is a potential therapeutic target in the prevention and treatment of diabetes and its related complications.

Key words: endothelial dysfunction, high-fat diet, inflammation, insulin resistance, muscle microvasculature, obesity.

INTRODUCTION

The prevalence of obesity and obesity-related diabetes has reached a global epidemic and chronic inflammation has been recognized as a key player in the development of insulin resistance and diabetes [1–4]. Growing evidence from both clinical and animal studies has suggested that endothelial dysfunction and vascular insulin resistance coexist in obesity and diabetes and they may play a causative role in the development of metabolic insulin resistance [5–9] in addition to accelerating diabetes-related cardiovascular complications [10,11]. This is particularly true in the setting of microvasculature as it provides endothelial exchange surface area for substrate exchanges and metabolic waste removal and thus profoundly affects cell metabolism, function and health. Muscle microvasculature is also insulin-responsive and ample evidence has confirmed that it has a critical role in regulating muscle insulin action [12–18]. In healthy humans or laboratory animals, insulin at physiological concentrations potently recruits muscle microvasculature and expands the endothelial exchange surface area, leading to increased muscle delivery and action of insulin [12,19]. These actions are impaired in humans with moderate obesity, humans receiving lipid infusion and in rodents with either genetic or experimentally induced insulin resistance [20–24]. The impairment of insulin's microvascular action has been implicated in the development of metabolic insulin resistance in muscle as insulin-mediated microvascular recruitment occurs earlier than insulin-stimulated muscle glucose uptake *in vivo* and blockade of the insulin's vasodilatory action reduces insulin-stimulated glucose disposal by up to 40% [25], and all metabolic insulin resistance inducers have been shown to cause microvascular insulin resistance as well [26,27]. However, the temporal relationship between

Abbreviations: Ach, acetylcholine; CEU, contrast-enhanced ultrasound; eNOS, endothelial nitric oxide synthase; ERK, extracellular-signal-regulated protein kinase; HFD, high-fat diet; IKK, Iκ B kinase; Iκ B, inhibitor of nuclear factor κB; NF-κB, nuclear factor κB; MBF, microvascular blood flow; MBV, microvascular blood volume; MFV, microvascular flow velocity.

Correspondence: Dr Zhenqi Liu (email zl3e@virginia.edu).

microvascular and metabolic insulin resistance has not been explored.

Obesity is associated with chronic inflammation which plays a key role in the pathogenesis of insulin resistance. Inhibitor of nuclear factor κB (IκB) kinase β (IKKβ) is a serine kinase that controls the activation of nuclear factor κB (NF-κB) and is the critical node linking inflammation to obesity-induced insulin resistance [28,29]. Activation of IKKβ leads to increased phosphorylation and degradation of IκB with subsequent NF-κB activation and cellular inflammatory responses, leading to impaired insulin signalling in both key metabolic organs (liver, adipose tissue and skeletal muscle) and vasculature in animal models of diet-induced obesity [5,28]. Inhibition of the NF-κB inflammation pathway using salicylate, a potent IKKβ inhibitor reverses obesity- and diet-induced insulin resistance, blocks lipid infusion-induced metabolic insulin resistance in laboratory animals [30,31], and improves glycaemic control in obese non-diabetic adults [32,33]. Our group has previously reported that salsalate attenuates non-esterified fatty acid-induced microvascular and metabolic insulin resistance in humans [34]. Whether inhibition of the inflammation pathway differentially affects insulin's vascular actions compared with metabolic actions has not been examined.

In the present study, we explored the temporal relationship among vascular inflammation, vascular and muscle metabolic insulin resistance in rats fed on a high-fat diet (HFD) for various durations ranging from 3 days to 4 weeks with or without simultaneous sodium salicylate treatment. Our results reveal that muscle microvascular insulin resistance is an early event in the development of diet-induced obesity and inhibition of the NF-κB pathway with sodium salicylate fully restores microvascular insulin responses and ameliorates metabolic insulin resistance in the setting of HFD feeding.

MATERIALS AND METHODS

Animal preparations and experimental protocols

Adult male Sprague–Dawley rats (~200–250 g, Charles River Laboratories) were placed on an HFD (60% calories from saturated fat, Research Diets) with or without administration of sodium salicylate (120 mg/kg/day) by oral gavage for 3 days, 1 week, 2 weeks or 4 weeks. At the dose selected, sodium salicylate potently inhibits NF-κB activity [31]. Additional rats were fed on a chow diet as controls. Rats were housed at 22 ± 2 °C, on a 12-h light/12-h dark cycle and given water *ad libitum*. After the feeding period, rats were starved overnight and anaesthetized with pentobarbital sodium [50 mg/kg i.p. (intraperitoneally), Abbott Laboratories]. They were then placed in a supine position on a heating pad to ensure euthermia and intubated to maintain a patent airway. The carotid artery and the jugular vein were cannulated with PE50 polyethylene tubing (Fisher Scientific) for blood pressure monitoring, arterial blood sampling and various infusions. After a 30–45 min baseline period to ensure haemodynamic stability and stable anaesthesia, rats received a 2-h euglycaemic–hyperinsulinaemic clamp (3 milliunits/kg/min). Arterial blood glucose was determined every 10 min using an Accu-Chek Advantage glucometer (Roche Diagnostics) and 30% dextrose was infused at a variable rate to maintain blood glucose within 10% of basal levels. Steady-state whole body glucose disposal rates were calculated. Hind-limb muscle microvascular blood volume (MBV), microvascular flow velocity (MFV) and microvascular blood flow (MBF) were determined at 0, 30, 60 and 120 min using contrast-enhanced ultrasound (CEU), as described previously [12,15]. Rats were then killed with anaesthetic overdose, gastrocnemius muscle and aorta were removed and freeze-clamped for later measurement of inflammation and insulin signalling molecules using Western blot analysis, as described below. Distal saphenous arteries were dissected immediately after the rats were killed and used for myograph studies.

Throughout the study, mean arterial blood pressure (MAP) was monitored via a sensor connected to the carotid arterial catheter (Harvard Apparatus and AD Instruments). Pentobarbital sodium was infused throughout the study at a variable rate to maintain steady levels of anaesthesia and blood pressure. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85-23, revised 1996) and the study protocols were approved by the Animal Care and Use Committee of the University of Virginia.

Measurement of plasma nitric oxide contents

Plasma nitric oxide (NO) levels were measured using a 280i Nitric Oxide Analyzer (GE Analytical Instruments), according to the manufacturer's instructions. In brief, ice-cold ethanol was mixed with plasma samples at a ratio of 2:1, kept at 0 °C for 30 min and then centrifuged at ~16 000 ***g*** for 5 min. The supernatant was then used for NO analysis based on a gas-phase chemiluminescent reaction between NO and ozone.

Determination of plasma cholesterol, triacylglycerol and insulin levels

Fasting total plasma cholesterol and triacylglycerol levels were determined using a DCL Total Cholesterol Assay Kit and Roche Diagnostics Triglyceride Assay. Plasma insulin concentrations were measured using a rat insulin ELISA assay kit (Mercodia AB).

Myograph studies

After removing the adhering connective tissue, distal saphenous artery was cut into segments of ~2 mm in length and vessel functions were measured as previously described [35]. Briefly, each segment was mounted in a Multi Myograph System (Danish Myo Technology). The organ chamber was filled with 6 ml of physiological salt solution buffer (130 mM NaCl, 4.7 mM KCl, 1.6 mM $CaCl_2$, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 14.9 mM $NaHCO_3$, 0.026 mM EDTA and 5.5 mM glucose, pH 7.4), which was constantly bubbled with 95% O_2/5% CO_2 and maintained at 37 °C. Each ring was stretched initially to an optimal tension (5 mN) and then allowed to stabilize at baseline tone. After pre-constriction with phenylephrine (2 μM), vasodilatory responses to acetylcholine (Ach), sodium nitroprusside (SNP) and insulin were recorded.

NF-κB DNA-binding activity

Fresh aorta tissues were used for determination of NF-κB activity as described in [36]. Nuclear extracts from aorta were obtained by using nuclear extraction kits (Active Motif). The DNA-binding activity of the NF-κB p65 subunit was determined by ELISA (Active Motif) according to the manufacturer's instructions.

Determination of protein expression and phosphorylation in muscle and aorta

Total eNOS (endothelial NO synthase), Akt (protein kinase B), ERK1/2 (extracellular-signal-regulated protein kinase 1 and 2) and β-actin and phosphorylation of eNOS, Akt, ERK1/2 and IκBα in gastrocnemius and/or aorta were determined using Western blot analysis, as previously described [12,37]. Primary antibodies against phospho-eNOS (Ser^{1177}), total eNOS, phospho-Akt (Ser^{473}), total Akt, phospho-ERK (Thr^{202}/Tyr^{204}) and total ERK1/2, β-actin and phospho-IκBα were purchased from Cell Signaling Technology. All blots were developed using ECL (GE Healthcare Bio-Sciences). Chemiluminescence blot images were captured using the UVP imaging system and quantified using ImageQuant 3.3 software. For protein phosphorylation analyses, both the total and the phospho-specific densities were quantified and the ratios of phospho-specific to total density were calculated.

Statistical analysis

All data are presented as means ± S.E.M. Statistical analyses were performed with SigmaStat 11.0 software (Systat Software), using either Student's *t* test or ANOVA with post-hoc analysis as appropriate. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Effect of HFD feeding on body weight gain and plasma parameters

Compared with chow diet, rats fed on an HFD gained a similar amount of weight and had similar fasting plasma glucose concentrations (Table 1). In rats fed on the HFD, fasting plasma levels of triacylglycerols, cholesterol and insulin increased steadily over the 4-week feeding period and were significantly higher at the end than those rats on a chow diet. Salicylate treatment did not significantly affect body weight gain but decreased fasting plasma triacylglycerol, cholesterol and insulin levels to the levels seen in chow-diet rats.

Effects of HFD and sodium salicylate on insulin and NF-κB signalling in aorta

We first examined the time course of insulin signalling in large conduit vessel (aorta) using insulin-mediated Akt, eNOS and ERK1/2 phosphorylation as indices (Figure 1). Insulin potently stimulated Akt phosphorylation in all control animals and in rats fed on the HFD for 3 days ($P < 0.05$ for all). However, insulin-mediated Akt phosphorylation was totally abolished in rats fed on the HFD for 1 week and beyond, which was reversed completely by salicylate administration. Insulin significantly increased aortic ERK phosphorylation and this effect was not affected by either HFD feeding or the combination of the HFD and salicylate. On the contrary, insulin effects on aortic eNOS phosphorylation followed exactly the same pattern as Akt phosphorylation in that HFD abolished insulin-stimulated eNOS phosphorylation after 1 week, but this was again prevented by salicylate treatment.

As prior study showed that aortic inflammation is an early event in diet-induced obesity [5], we determined aortic IκBα phosphorylation (a surrogate of IKKβ activation) and NF-κB DNA-binding activity. As shown in Figures 1(G), 1(F) and 1(I), HFD progressively increased aortic IκBα phosphorylation and p65 DNA-binding activity ($P < 0.05$, ANOVA) and sodium salicylate treatment effectively restored these inflammatory responses to the control levels.

HFD induces endothelial dysfunction and insulin resistance in small resistance arteries which can be prevented by sodium salicylate

We next examined *ex vivo* the time course of endothelial dysfunction and insulin resistance induced by HFD feeding and whether sodium salicylate treatment can prevent their occurrence in isolated distal saphenous arteries (Figure 2). The HFD did not induce a significant impairment in the vasodilatory responses to Ach until week 4 and this impairment was reversed by administration of sodium salicylate (Figures 2A–2D). Similarly, the vasodilatory response to insulin remained unaffected until week 4 and again simultaneous administration of sodium salicylate improved insulin responses almost back to the levels seen in controls (Figures 2E–2H). Thus, resistance artery insulin resistance lags behind that in large conduit artery aorta.

Time course of HFD and sodium salicylate on microvascular insulin responses

Previous evidence suggests that microvascular recruitment is an early action of insulin which occurs much sooner than insulin-mediated muscle glucose uptake [12] and accounts for up to 40% of insulin-stimulated glucose disposal during insulin infusion [25]. We thus determined the time course of microvascular insulin resistance development in the setting of HFD feeding. As shown in Figure 3, insulin potently recruited muscle microvasculature as evidenced by a significant increase in muscle MBV in all control animals (∼60%, $P < 0.05$). This effect was blunted by HFD feeding in as little as 3 days and abolished at 1 week and beyond ($P < 0.05$ for all). Simultaneous administration of sodium salicylate fully restored muscle microvascular insulin responses. Neither HFD feeding nor HFD and sodium salicylate treatment altered muscle MFV. As such, muscle MBF followed a similar pattern (Figure 4).

As insulin-stimulated microvascular recruitment is NO-dependent, we determined plasma NO contents before and after insulin clamps in all groups (Figure 5). Insulin infusion potently increased plasma NO contents in all control groups. Although HFD feeding did not alter basal NO levels, it blunted insulin-stimulated increase in NO production in as little as 3 days and totally abolished it after 1 week, a pattern that is similar to microvascular responses in HFD animals (Figures 3 and 4). Sodium

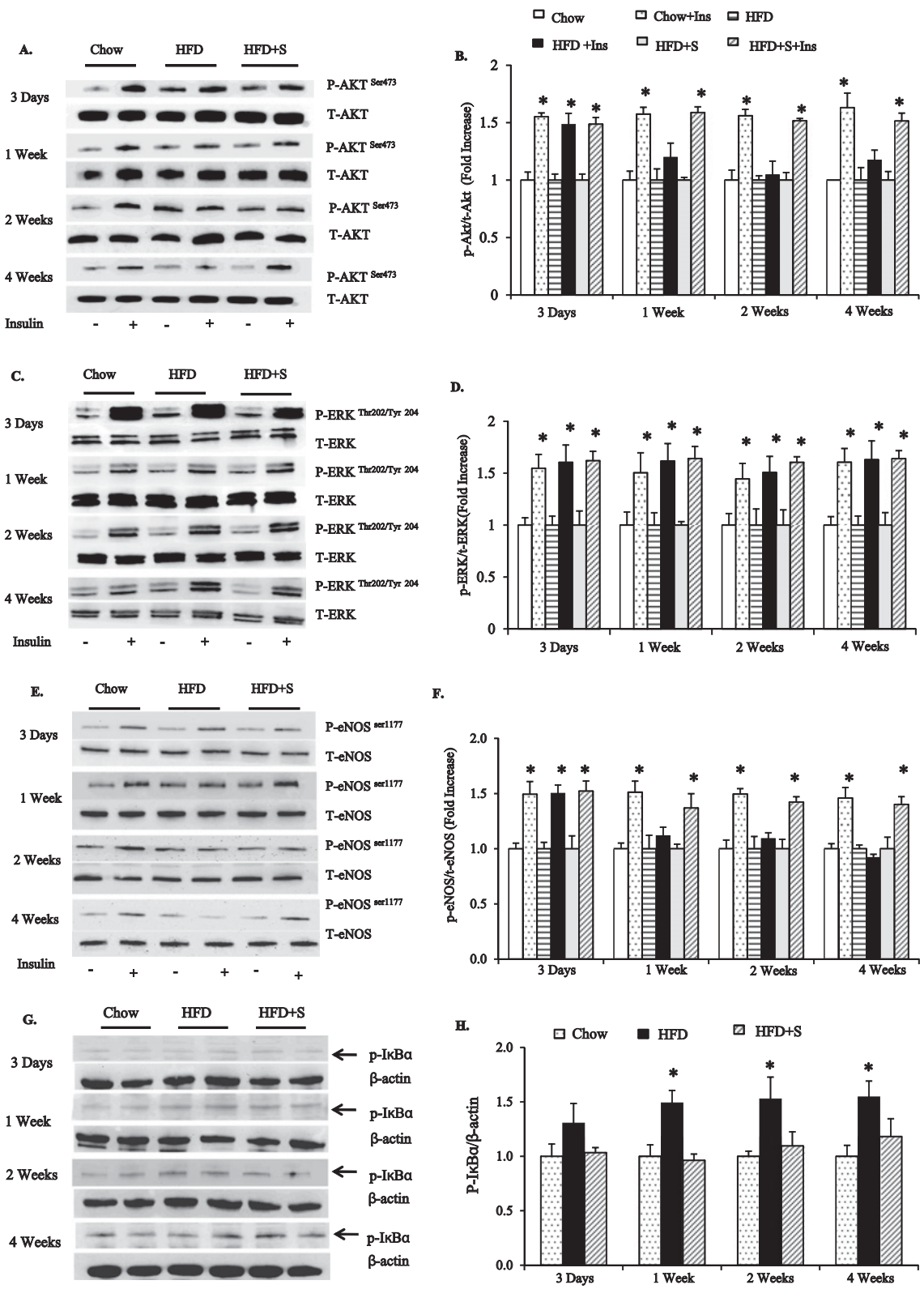

Figure 1 **Effects of HFD ± sodium salicylate on insulin and NF-κB signalling in aorta**
(**A** and **B**) Akt phosphorylation, (**C** and **D**) ERK1/2 phosphorylation, (**E** and **F**) eNOS phosphorylation, (**G** and **H**) IκBα phosphorylation and (**I**) NF-κB DNA-binding activity. (**A**, **C**, **E** and **G**) Representative Western blots; (**B**, **D**, **F** and **H**) quantitative measurements. Compared with respective control without insulin, $*P < 0.05$. $n = 3$–4.

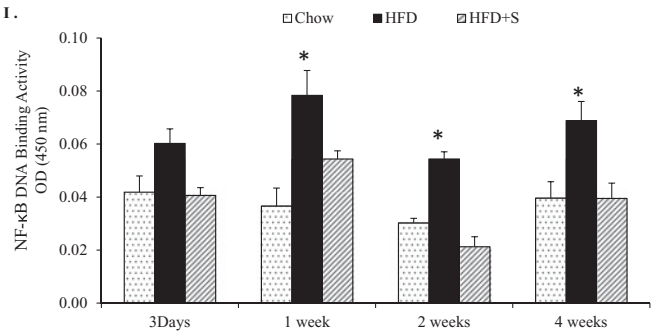

Figure 1 Continued

Table 1 Body weight gain and fasting plasma parameters
Values are means ± S.E.M.; HFD + S: HFD + salicylate. n=5–6.

		3 Days	1 Week	2 Weeks	4 Weeks
Body weight gain (g)	Chow	27.6 ± 3.2	64.0 ± 5.4	135.4 ± 6.6	218.4 ± 9.5
	HFD	28.1 ± 3.1	65.6 ± 8.4	151.4 ± 12.7	239.2 ± 10.9
	HFD + S	28.6 ± 3.1	53.6 ± 3.4	118.3 ± 7.7	203.7 ± 11.2
Glucose (mmol/l)	Chow	4.79 ± 0.09	4.53 ± 0.08	4.92 ± 0.09	4.99 ± 0.10
	HFD	4.76 ± 0.09	4.60 ± 0.13	4.78 ± 0.24	4.73 ± 0.16
	HFD + S	4.92 ± 0.12	4.32 ± 0.12	4.63 ± 0.15	4.67 ± 0.08
Triacylglycerols (mmol/l)	Chow	0.46 ± 0.06	0.47 ± 0.08	0.53 ± 0.02	0.50 ± 0.03
	HFD	0.49 ± 0.05	0.50 ± 0.05	0.53 ± 0.02	0.56 ± 0.04*
	HFD + S	0.45 ± 0.04	0.51 ± 0.03	0.50 ± 0.02	0.55 ± 0.06
Total cholesterol (mmol/l)	Chow	0.99 ± 0.05	1.19 ± 0.06	1.11 ± 0.15	1.37 ± 0.13
	HFD	0.99 ± 0.07	1.13 ± 0.06	1.23 ± 0.08	1.59 ± 0.19*
	HFD + S	1.04 ± 0.07	1.20 ± 0.05	1.06 ± 0.08	1.44 ± 0.10
Insulin (pmol/l)	Chow	87.6 ± 6.52	83.8 ± 6.1	94.4 ± 5.9	102.0 ± 10.9
	HFD	90.3 ± 11.4	93.2 ± 8.2	125.1 ± 12.3	139.8 ± 7.5*
	HFD + S	97.2 ± 6.0	103.1 ± 6.6	128.1 ± 9.8	127.2 ± 6.8

*$P < 0.05$ compared with chow.

salicylate treatment did not change basal plasma NO contents but fully restored insulin-stimulated NO production in all HFD-fed rats, again a pattern paralleling the improved microvascular insulin responses in these rats.

Time course of HFD and sodium salicylate on muscle metabolic insulin responses

Finally, we assessed muscle metabolic insulin sensitivity by calculating glucose infusion rate during the insulin clamps and Western blot analysis of Akt and ERK1/2 phosphorylation in muscle in all study animals. As shown in Figure 6, compared with chow-fed controls, insulin-stimulated glucose disposal did not change significantly before day 3 but then declined significantly and progressively afterwards (by ∼20% at week 1, ∼40% at week 2 and ∼60% at week 4, $P < 0.05$ for all) in HFD-fed rats. Animals that received the HFD and sodium salicylate exhibited a marked improvement in insulin-stimulated glucose disposal by week 2 (∼50% increase) and week 4 (∼70% increase; $P < 0.05$ for both). Interestingly, neither HFD nor HFD plus sodium salicylate affected insulin-mediated Akt or ERK1/2 phosphorylation in muscle (Figure 7).

DISCUSSION

Insulin resistance is a key feature of obesity-related diabetes and develops in multiple organs, including skeletal muscle and vasculature. Evidence thus far has confirmed that vascular dysfunction and insulin resistance coexist and both contribute to the pathogenesis of diabetes and its cardiovascular complications. Furthermore, muscle microvasculature plays a pivotal role in the regulation of muscle insulin delivery and action. In the present study, we explored the temporal relationship between vascular inflammation, vascular insulin resistance and metabolic insulin resistance in rats fed on an HFD for various durations ranging from 3 days to 4 weeks, using a combination of euglycaemic–hyperinsulinaemic clamp, Western blotting, myograph study and CEU techniques. We found that microvascular insulin resistance is an early event in diet-induced obesity and insulin resistance

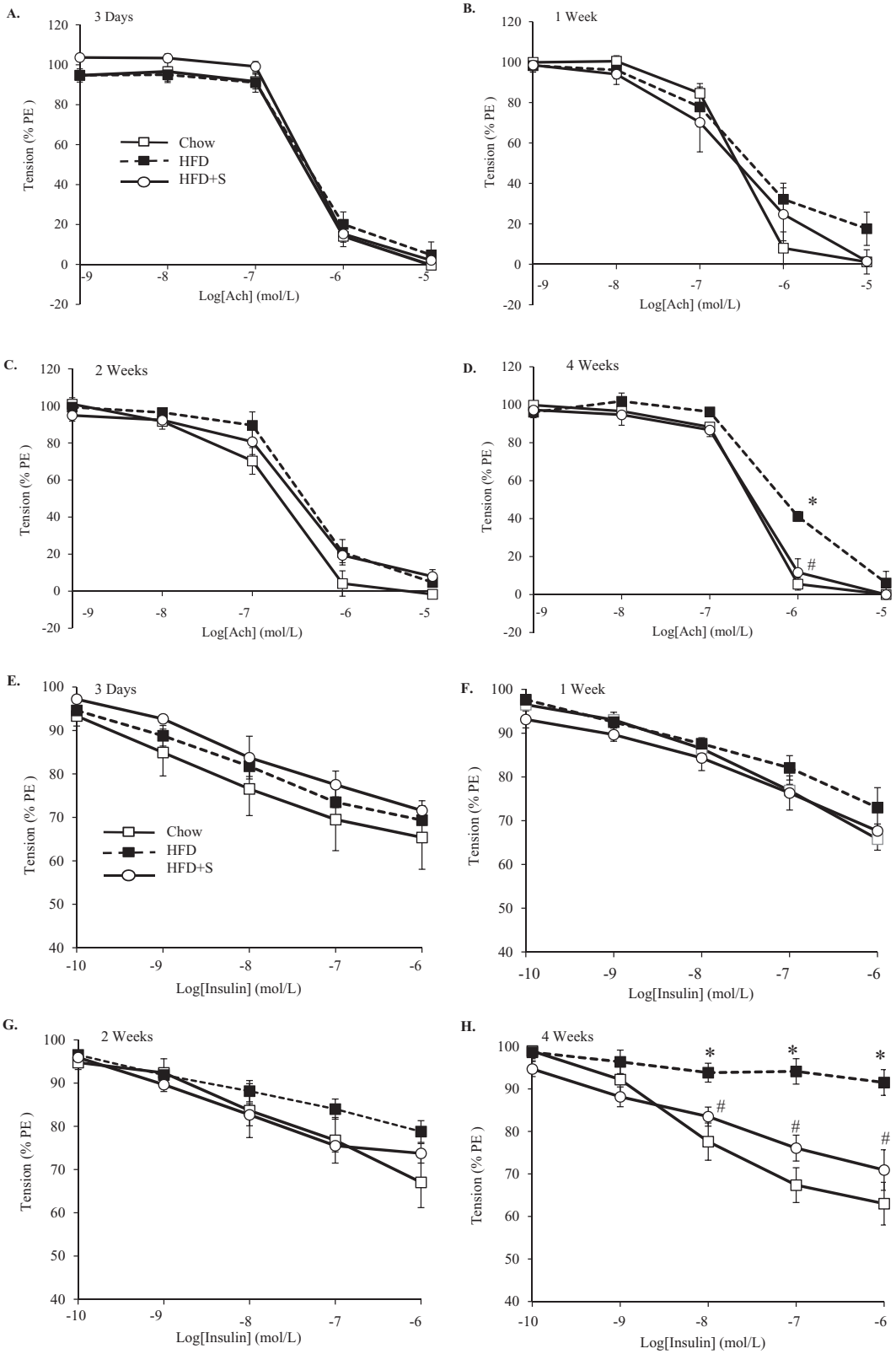

Figure 2 Effects of HFD ± sodium salicylate on resistance arterial endothelial function and insulin responses

Vasodilatory responses to Ach (**A**–**D**) and insulin (**E**–**H**) were determined using distal saphenous arteries that were dissected from rats fed on either chow or an HFD for 3 days (**A**), 1 week (**B**), 2 weeks (**C**) and 4 weeks (**D**). Compared with chow, $*P < 0.05$. Compared with HFD, $\#P < 0.05$. $n = 5$–6.

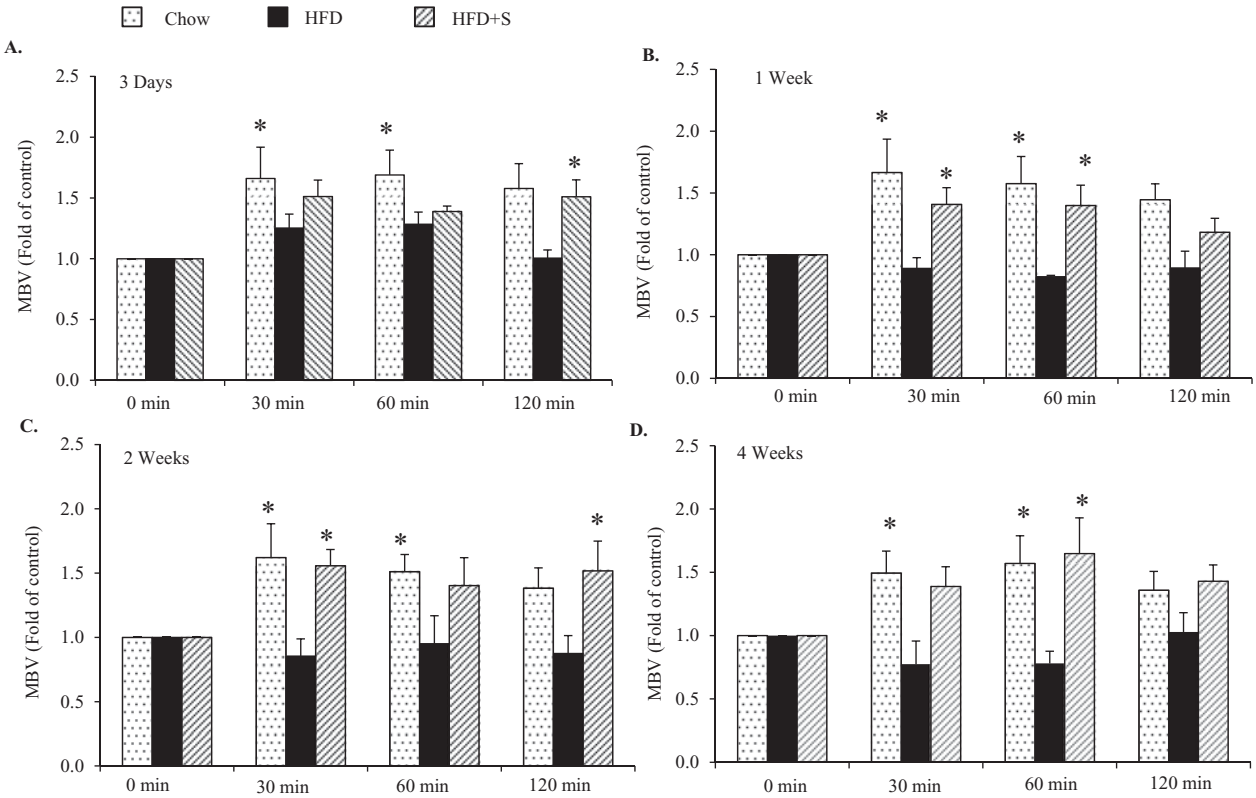

Figure 3 **Time course effect of HFD ± sodium salicylate on insulin-mediated changes in muscle MBV**
Each rat received a 2-h euglycaemic–hyperinsulinaemic clamp after an HFD for 3 days (**A**), 1 week (**B**), 2 weeks (**C**) and 4 weeks (**D**) with or without simultaneous sodium salicylate treatment. MBV was measured using CEU at baseline and 30, 60 and 120 min after the initiation of insulin infusion. Rats fed normal chow were used as a control. Compared with respective baseline (0 min), $*P < 0.05$. $n = 5$–6.

and inhibition of the NF-κB inflammation pathway with sodium salicylate effectively reduces vascular inflammation, completely restores vascular insulin responses and significantly improves metabolic insulin sensitivity in HFD-fed rodents. Thus, our results clearly indicate that microvasculature is an early responder to inflammation during the development of diet-induced insulin resistance and obesity.

Our observation that during HFD feeding insulin-stimulated Akt and eNOS phosphorylation did not decrease in aorta until 1 week and insulin-induced resistance vessel relaxation was not impaired until 4 weeks but microvascular insulin resistance occurred in as little as 3 days suggests that the timeline of vascular insulin resistance development varies among different arterial segments and microvasculature is more susceptible to inflammatory insult. However, several caveats exist. First, aorta and small resistance vessels include both endothelial cells and vascular smooth muscle cells, whereas microvasculature is mostly lined with only endothelial cells. Secondly, different techniques were used to assess insulin responses in different arterial segments. Certainly, it would be ideal to use the same technique to assess insulin responses but it is technically impossible and physiologically irrelevant given different arterial segments have different structures and functions. That the pattern changes were consistent within each vascular segments, i.e. the changes in aortic Akt and eNOS phosphorylation, the *ex vivo* vasodilatory responses to Ach and insulin in isolated distal saphenous artery and the changes in muscle MBV and MBF with those of plasma NO contents strongly argues that the variation in insulin resistance timeline among arterial segments is real.

Previous studies have confirmed that microvasculature critically regulates insulin's delivery to and action in muscle and expansion of muscle MBV induced by factors such as adiponectin, angiontensin-(1–7), glucagon-like peptide 1, losartan and muscle contraction are associated with increased muscle insulin action [14,15,17,38,39]. In contrast, factors causing metabolic insulin resistance are also able to induce microvascular insulin resistance [26,27]. Our observation that microvascular insulin resistance occurs earlier than muscle metabolic insulin resistance is intriguing and suggests that the former may contribute to the pathogenesis of the latter. Our results are clearly consistent with a prior report demonstrating that HFD feeding caused insulin resistance in aorta in 1 week but it took those mice 4–8 weeks to develop insulin resistance in muscle, liver and adipose tissue [5]. Indeed, in C57BL/6 mice fed on an HFD for 1.5, 3, 6 and 20 weeks, glucose infusion rates and muscle glucose uptake did not decrease significantly until week 3 despite both leptin and resistin being elevated in week 1.5 [40]. In a different study also conducted in HFD-fed mice, muscle glucose uptake

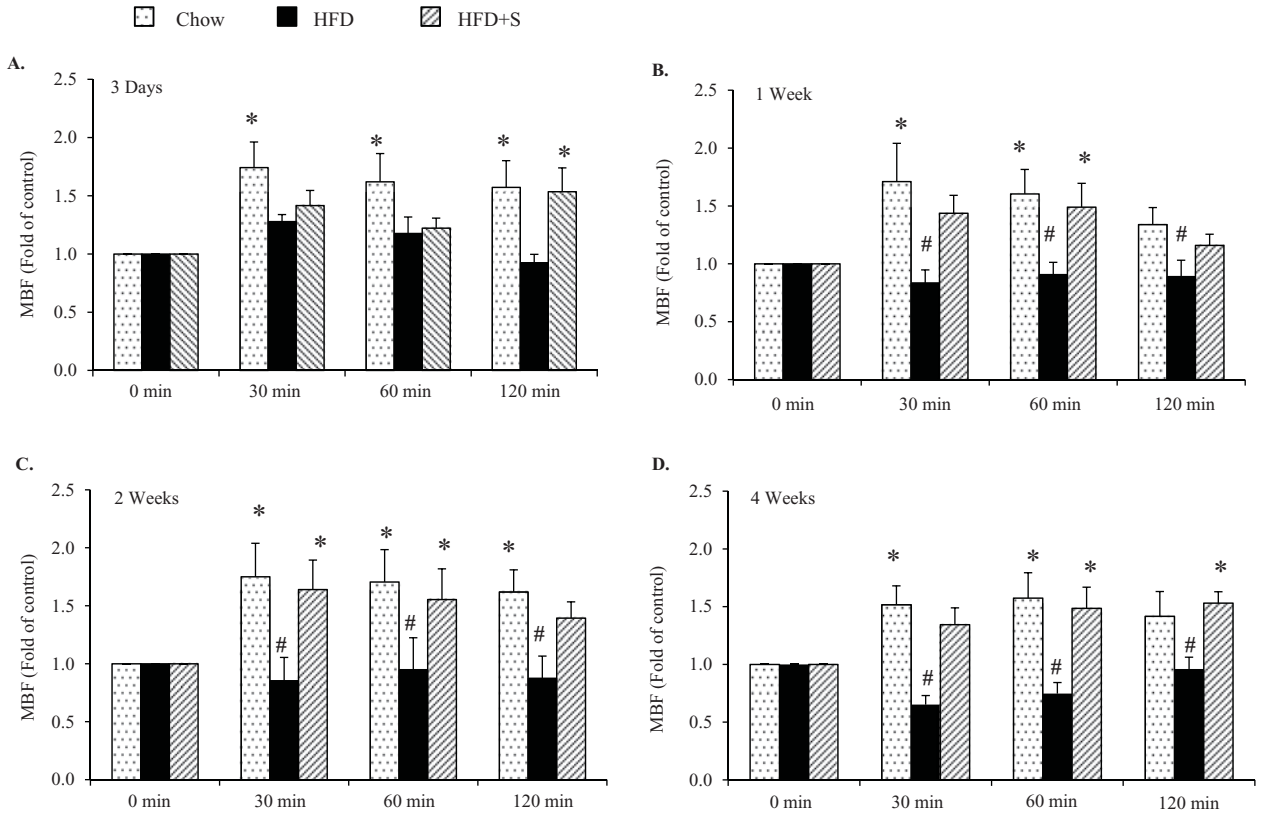

Figure 4 **Time course effect of HFD ± sodium salicylate on insulin-mediated changes in muscle MBF**
Each rat received a 2-h euglycaemic–hyperinsulinaemic clamp after an HFD for 3 days (**A**), 1 week (**B**), 2 weeks (**C**) and 4 weeks (**D**) with or without simultaneous salicylate treatment. MBF was calculated at baseline and 30, 60 and 120 min after the initiation of insulin infusion. Rats fed normal chow were used as a control. Compared with respective baseline (0 min), $*P < 0.05$. Compared with HFD at the same time point, $\#P < 0.05$. $n = 5–6$.

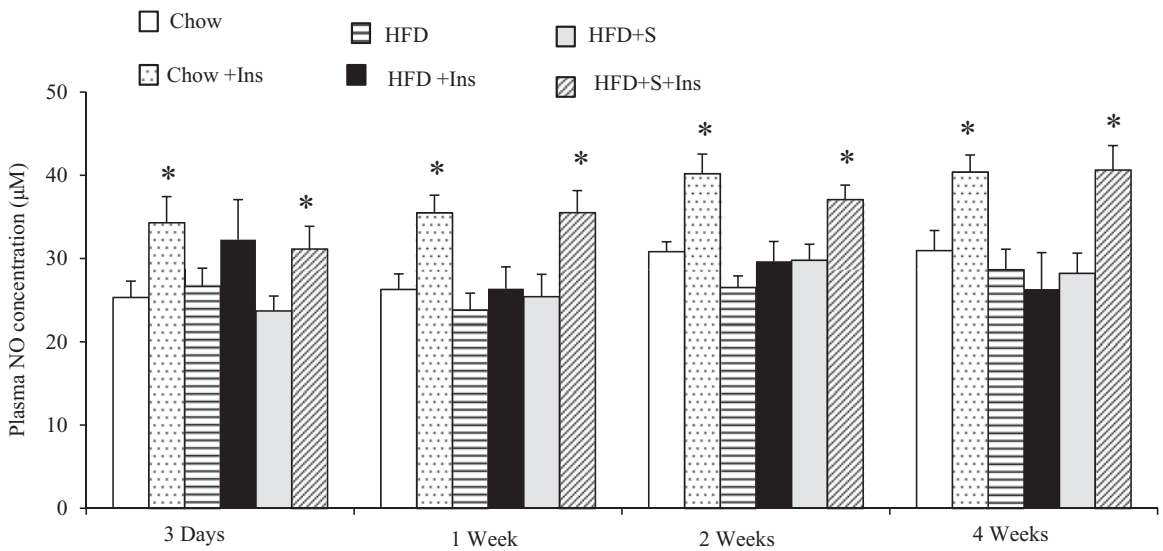

Figure 5 **Effects of HFD ± sodium salicylate on plasma NO contents**
Compared with respective control without insulin, $*P < 0.05$. $n = 5–6$.

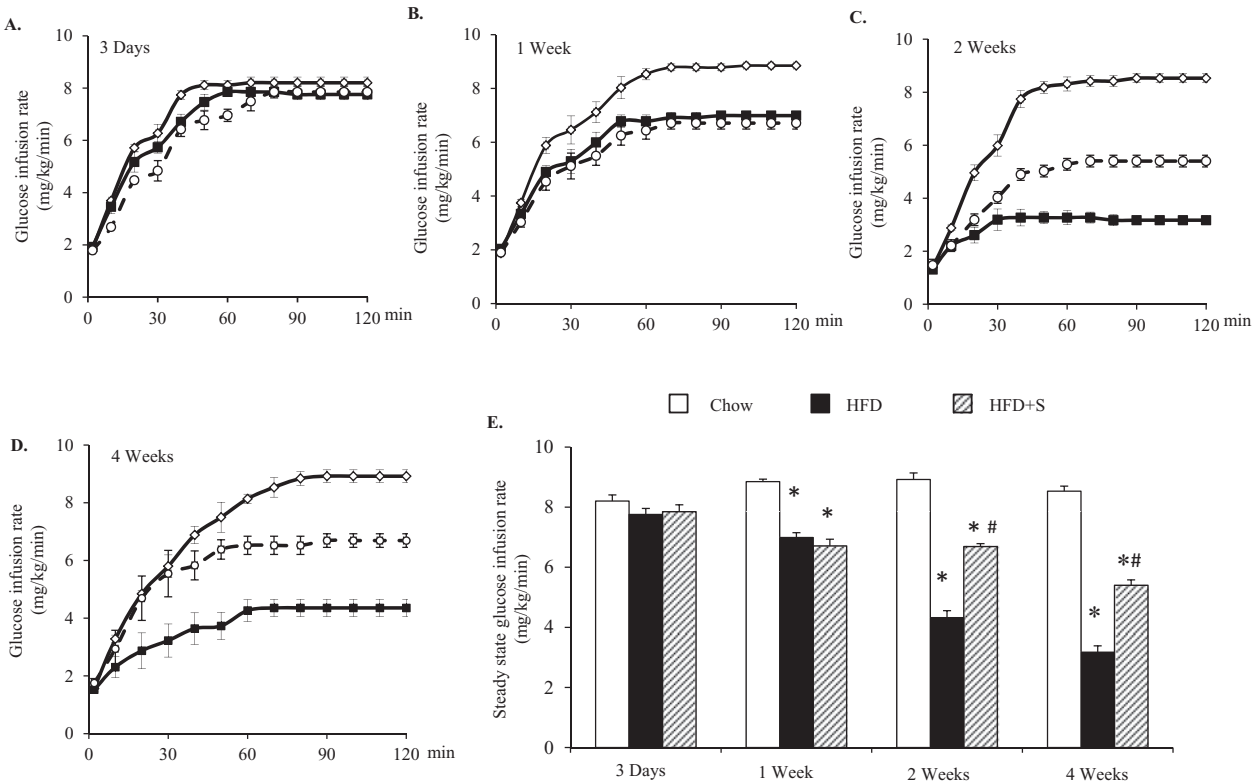

Figure 6 Time course effect of HFD ± sodium salicylate on metabolic insulin sensitivity

Each rat received a 2-h euglycaemic–hyperinsulinaemic clamp after an HFD for 3 days (**A**), 1 week (**B**), 2 weeks (**C**) and 4 weeks (**D**) with or without simultaneous sodium salicylate treatment and the glucose infusion rates (GIR) were recorded. Compared with baseline, $*P < 0.05$. Compared with HFD, $\#P < 0.05$. $n = 5$–6.

again did not decrease until week 3 [41]. Thus, our results firmly place the vascular endothelium at the forefront of the inflammation and insulin-resistance rampage.

Sodium salicylate, by specifically binding to IKKβ, reduces ATP binding and thus inhibits IKKβ activity which leads to increased IκB phosphorylation and degradation and translocation of NF-κB to the nucleus [30]. In the present study, we observed that sodium salicylate potently inhibited HFD-induced IκBα phosphorylation and completely restored microvascular insulin responses, insulin-mediated Akt and eNOS phosphorylation in aorta and vasodilatory responses to insulin in the resistance artery. Together with prior findings in diet-induced obesity mice that it took 1 week for IκBα phosphorylation to increase in aorta, 8 weeks in liver and muscle and 14 weeks in adipose tissue [5], our results confirm an essential role of inflammation in the pathogenesis of vascular insulin resistance in all segments of the arterial tree. It is worth mentioning that c-Jun N-terminal kinase (JNK), another key player in obesity-induced inflammation, may also contribute to obesity-induced vascular inflammation and insulin resistance which warrant further investigation [42].

Consistent with prior studies showing that treatment of rodents with salicylates attenuates obesity- and diet-induced insulin resistance in muscle [31,43], we found in the present study that metabolic insulin resistance improved significantly with sodium salicylate treatment, as evidenced by a marked increase in insulin-mediated glucose disposal. That muscle metabolic insulin responses were not fully restored despite a complete restoration of vascular insulin responses is consistent with previous evidence that the maximal contribution of microvasculature to insulin-mediated metabolic effect (glucose disposal) is <40% [25]. This is certainly in agreement with clinical trials showing that salsalate treatment improves glycaemia and insulin-mediated glucose disposal in obese and diabetic humans without restoring plasma glucose concentrations back to normal in patients with Type 2 diabetes mellitus (T2DM) [44–46].

It is of interest to note that HFD decreased aortic eNOS and Akt phosphorylation but not muscle Akt phosphorylation despite a marked decrease in glucose infusion rates. This is not surprising as prior studies have shown that Akt is not the critical node in muscle insulin resistance. Insulin activation of Akt isoforms is normal in muscle of obese non-diabetic and diabetic subjects despite a marked decrease in IRS-1- and IRS-2-associated phosphoinositide 3-kinase (PI3K) activities (~50% and 40% respectively) and in insulin-stimulated glucose disposal (~60%) in obese diabetic subjects [47]. In mice fed on an HFD for either 1 or 3 weeks, insulin-stimulated Akt phosphorylation in liver, adipose tissue and muscle was as same as that in chow-fed rats [41]. Similarly, we have previously observed in lipid-infused rats insulin-stimulated muscle Akt phosphorylation was similar between the control and lipid-infusion groups despite

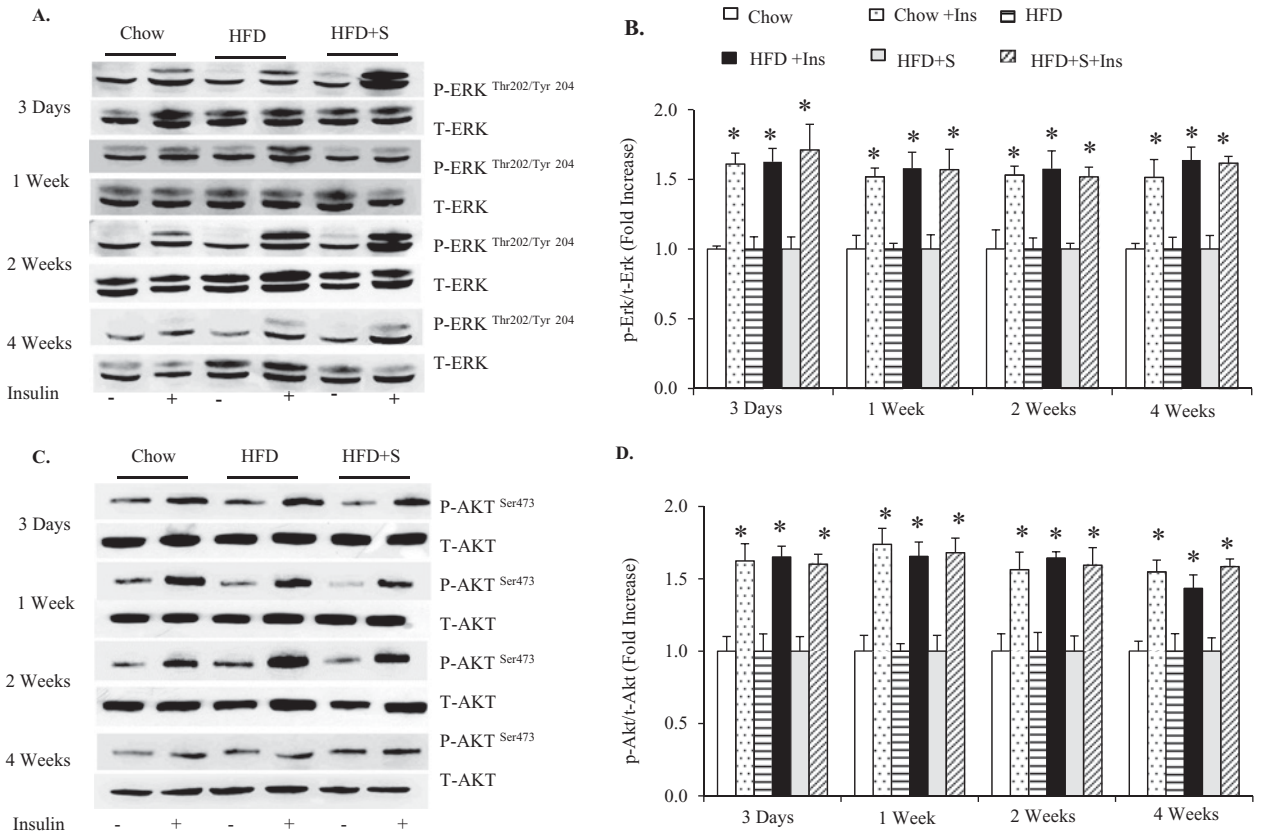

Figure 7 **Effects of HFD ± sodium salicylate on skeletal muscle insulin signalling**
(**A**) Akt phosphorylation; (**B**) ERK1/2 phosphorylation. Compared with respective control without insulin, $*P < 0.05$. $n = 3$.

a marked decrease in insulin-mediated glucose disposal in the latter [38].

In conclusion, our results demonstrate that microvascular insulin resistance is an early event in diet-induced obesity and inflammation plays an essential role in this process. As muscle microvasculature critically regulates muscle insulin delivery and action, microvascular insulin resistance may play a causative role in the development of muscle metabolic insulin resistance and early intervention aimed at reducing microvascular insulin resistance may help prevent and/or treatment diabetes and its associated complications.

CLINICAL PERSPECTIVES

- Evidence from both clinical and animal studies has confirmed the presence of endothelial dysfunction and insulin resistance in muscle microvasculature in obesity and diabetes which may play a causative role in the development of metabolic insulin resistance.
- The present study explores the temporal relationship between microvascular and metabolic insulin resistance and shows that inflammation-induced microvascular insulin resistance is an early event in diet-induced obesity and plays a causative role in the development of metabolic insulin resistance.
- These findings help us better understand the pathogenesis of obesity-associated insulin resistance and are of physiological as well as clinical importance as microvasculature controls the delivery of oxygen, nutrient and hormones into tissue and their exchanges between plasma and tissue interstitium by providing an endothelial exchange surface area. Thus, early intervention aimed at reducing microvascular insulin resistance may help to prevent and/or treat diabetes and its associated complications.

AUTHOR CONTRIBUTION

Lina Zhao, Zhuo Fu, Jing Wu, Kevin Aylor and Zhenqi Liu researched data. Eugene Barrett and Wenhong Cao contributed to the discussion. Lina Zhao and Zhenqi Liu wrote the manuscript.

FUNDINGS

This work was supported by the American Diabetes Association [grant numbers 1-11-CR-30 and 1-15-CE-32 (to Z.L.)] and the National Institutes of Health [grant numbers R01HL094722 and R01DK102359 (to Z.L.); and T32-DK07646 (to the University of Virginia)].

REFERENCES

1 Caballero, B. (2007) The global epidemic of obesity: an overview. Epidemiol. Rev. **29**, 1–5 CrossRef PubMed
2 Stein, C.J. and Colditz, G.A. (2004) The epidemic of obesity. J. Clin. Endocrinol. Metab. **89**, 2522–2525 CrossRef PubMed
3 Dandona, P., Aljada, A. and Bandyopadhyay, A. (2004) Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol. **25**, 4–7 CrossRef PubMed
4 Shoelson, S.E., Herrero, L. and Naaz, A. (2007) Obesity, inflammation, and insulin resistance. Gastroenterology **132**, 2169–2180 CrossRef PubMed
5 Kim, F., Pham, M., Maloney, E., Rizzo, N.O., Morton, G.J., Wisse, B.E., Kirk, E.A., Chait, A. and Schwartz, M.W. (2008) Vascular inflammation, insulin resistance, and reduced nitric oxide production precede the onset of peripheral insulin resistance. Arterioscler. Thromb. Vasc. Biol. **28**, 1982–1988 CrossRef PubMed
6 Caballero, A.E., Bousquet-Santos, K., Robles-Osorio, L., Montagnani, V., Soodini, G., Porramatikul, S., Hamdy, O., Nobrega, A.C. and Horton, E.S. (2008) Overweight Latino children and adolescents have marked endothelial dysfunction and subclinical vascular inflammation in association with excess body fat and insulin resistance. Diabetes Care **31**, 576–582 CrossRef PubMed
7 Caballero, A.E. (2003) Endothelial dysfunction in obesity and insulin resistance: a road to diabetes and heart disease. Obes. Res. **11**, 1278–1289 CrossRef PubMed
8 Jonk, A.M., Houben, A.J., de Jongh, R.T., Serne, E.H., Schaper, N.C. and Stehouwer, C.D. (2007) Microvascular dysfunction in obesity: a potential mechanism in the pathogenesis of obesity-associated insulin resistance and hypertension. Physiology **22**, 252–260 CrossRef PubMed
9 Muris, D.M., Houben, A.J., Schram, M.T. and Stehouwer, C.D. (2013) Microvascular dysfunction: an emerging pathway in the pathogenesis of obesity-related insulin resistance. Rev. Endocr. Metab. Disord. **14**, 29–38 CrossRef PubMed
10 van Sloten, T.T., Henry, R.M., Dekker, J.M., Nijpels, G., Unger, T., Schram, M.T. and Stehouwer, C.D. (2014) Endothelial dysfunction plays a key role in increasing cardiovascular risk in type 2 diabetes: the Hoorn study. Hypertension **64**, 1299–1305 CrossRef PubMed
11 Steinberger, J. and Daniels, S.R. (2003) Obesity, insulin resistance, diabetes, and cardiovascular risk in children: an American Heart Association scientific statement from the Atherosclerosis, Hypertension, and Obesity in the Young Committee (Council on Cardiovascular Disease in the Young) and the Diabetes Committee (Council on Nutrition, Physical Activity, and Metabolism). Circulation **107**, 1448–1453 CrossRef PubMed
12 Vincent, M.A., Clerk, L.H., Lindner, J.R., Klibanov, A.L., Clark, M.G., Rattigan, S. and Barrett, E.J. (2004) Microvascular recruitment is an early insulin effect that regulates skeletal muscle glucose uptake *in vivo*. Diabetes **53**, 1418–1423 CrossRef PubMed
13 Chai, W., Wang, W., Dong, Z., Cao, W. and Liu, Z. (2011) Angiotensin II receptors modulate muscle microvascular and metabolic responses to insulin *in vivo*. Diabetes **60**, 2939–2946 CrossRef PubMed
14 Chai, W., Zhang, X., Barrett, E.J. and Liu, Z. (2014) Glucagon-like peptide 1 recruits muscle microvasculature and improves insulin's metabolic action in the presence of insulin resistance. Diabetes **63**, 2788–2799 CrossRef PubMed
15 Zhao, L., Chai, W., Fu, Z., Dong, Z., Aylor, K.W., Barrett, E.J., Cao, W. and Liu, Z. (2013) Globular adiponectin enhances muscle insulin action via microvascular recruitment and increased insulin delivery. Circ. Res. **112**, 1263–1271 CrossRef PubMed
16 Fu, Z., Zhao, L., Chai, W., Dong, Z., Cao, W. and Liu, Z. (2013) Ranolazine recruits muscle microvasculature and enhances insulin action in rats. J. Physiol. **591**, 5235–5249 CrossRef PubMed
17 Fu, Z., Zhao, L., Aylor, K.W., Carey, R.M., Barrett, E.J. and Liu, Z. (2014) Angiotensin-(1–7) recruits muscle microvasculature and enhances insulin's metabolic action via mas receptor. Hypertension **63**, 1219–1227 CrossRef PubMed
18 Zhang, L., Vincent, M.A., Richards, S.M., Clerk, L.H., Rattigan, S., Clark, M.G. and Barrett, E.J. (2004) Insulin sensitivity of muscle capillary recruitment *in vivo*. Diabetes **53**, 447–453 CrossRef PubMed
19 Meijer, R.I., De Boer, M.P., Groen, M.R., Eringa, E.C., Rattigan, S., Barrett, E.J., Smulders, Y.M. and Serne, E.H. (2012) Insulin-induced microvascular recruitment in skin and muscle are related and both are associated with whole-body glucose uptake. Microcirculation **19**, 494–500 CrossRef PubMed
20 Liu, Z., Liu, J., Jahn, L.A., Fowler, D.E. and Barrett, E.J. (2009) Infusing lipid raises plasma free fatty acids and induces insulin resistance in muscle microvasculature. J. Clin. Endocrinol. Metab. **94**, 3543–3549 CrossRef PubMed
21 Liu, J., Jahn, L.A., Fowler, D.E., Barrett, E.J., Cao, W. and Liu, Z. (2011) Free fatty acids induce insulin resistance in both cardiac and skeletal muscle microvasculature in humans. J. Clin. Endocrinol. Metab. **96**, 438–446 CrossRef PubMed
22 Clerk, L.H., Vincent, M.A., Jahn, L.A., Liu, Z., Lindner, J.R. and Barrett, E.J. (2006) Obesity blunts insulin-mediated microvascular recruitment in human forearm muscle. Diabetes **55**, 1436–1442 CrossRef PubMed
23 Eggleston, E.M., Jahn, L.A. and Barrett, E.J. (2013) Early microvascular recruitment modulates subsequent insulin-mediated skeletal muscle glucose metabolism during lipid infusion. Diabetes Care **36**, 104–110 CrossRef PubMed
24 Wallis, M.G., Wheatley, C.M., Rattigan, S., Barrett, E.J., Clark, A.D. and Clark, M.G. (2002) Insulin-mediated hemodynamic changes are impaired in muscle of Zucker obese rats. Diabetes **51**, 3492–3498 CrossRef PubMed
25 Vincent, M.A., Barrett, E.J., Lindner, J.R., Clark, M.G. and Rattigan, S. (2003) Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin. Am. J. Physiol. Endocrinol. Metab. **285**, E123–E129 CrossRef PubMed
26 Clerk, L.H., Rattigan, S. and Clark, M.G. (2002) Lipid infusion impairs physiologic insulin-mediated capillary recruitment and muscle glucose uptake *in vivo*. Diabetes **51**, 1138–1145 CrossRef PubMed
27 Youd, J.M., Rattigan, S. and Clark, M.G. (2000) Acute impairment of insulin-mediated capillary recruitment and glucose uptake in rat skeletal muscle *in vivo* by TNF-alpha. Diabetes **49**, 1904–1909 CrossRef PubMed
28 Arkan, M.C., Hevener, A.L., Greten, F.R., Maeda, S., Li, Z.W., Long, J.M., Wynshaw-Boris, A., Poli, G., Olefsky, J. and Karin, M. (2005) IKK-beta links inflammation to obesity-induced insulin resistance. Nat. Med. **11**, 191–198 CrossRef PubMed
29 Shoelson, S.E., Lee, J. and Goldfine, A.B. (2006) Inflammation and insulin resistance. J. Clin. Invest. **116**, 1793–1801 CrossRef PubMed
30 Yin, M.J., Yamamoto, Y. and Gaynor, R.B. (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature **396**, 77–80 CrossRef PubMed
31 Yuan, M., Konstantopoulos, N., Lee, J., Hansen, L., Li, Z.W., Karin, M. and Shoelson, S.E. (2001) Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science **293**, 1673–1677 CrossRef PubMed
32 Goldfine, A.B., Silver, R., Aldhahi, W., Cai, D., Tatro, E., Lee, J. and Shoelson, S.E. (2008) Use of salsalate to target inflammation in the treatment of insulin resistance and type 2 diabetes. Clin. Transl. Sci. **1**, 36–43 CrossRef PubMed
33 Goldfine, A.B., Conlin, P.R., Halperin, F., Koska, J., Permana, P., Schwenke, D., Shoelson, S.E. and Reaven, P.D. (2013) A randomised trial of salsalate for insulin resistance and cardiovascular risk factors in persons with abnormal glucose tolerance. Diabetologia **56**, 714–723 CrossRef PubMed

34 Chai, W., Liu, J., Jahn, L.A., Fowler, D.E., Barrett, E.J. and Liu, Z. (2011) Salsalate attenuates free fatty acid-induced microvascular and metabolic insulin resistance in humans. Diabetes Care **34**, 1634–1638 CrossRef PubMed

35 Dong, Z., Chai, W., Wang, W., Zhao, L., Fu, Z., Cao, W. and Liu, Z. (2013) Protein kinase A mediates glucagon-like peptide 1-induced nitric oxide production and muscle microvascular recruitment. Am. J. Physiol. Endocrinol. Metab. **304**, E222–E228 CrossRef PubMed

36 Zhang, W.J., Wei, H., Hagen, T. and Frei, B. (2007) Alpha-lipoic acid attenuates LPS-induced inflammatory responses by activating the phosphoinositide 3-kinase/Akt signaling pathway. Proc. Natl. Acad. Sci. U.S.A. **104**, 4077–4082 CrossRef PubMed

37 Chai, W., Wu, Y., Li, G., Cao, W., Yang, Z. and Liu, Z. (2008) Activation of p38 mitogen-activated protein kinase abolishes insulin-mediated myocardial protection against ischemia-reperfusion injury. Am. J. Physiol. Endocrinol. Metab. **294**, E183–E189 CrossRef PubMed

38 Wang, N., Chai, W., Zhao, L., Tao, L., Cao, W. and Liu, Z. (2013) Losartan increases muscle insulin delivery and rescues insulin's metabolic action during lipid infusion via microvascular recruitment. Am. J. Physiol. Endocrinol. Metab. **304**, E538–E545 CrossRef PubMed

39 Inyard, A.C., Clerk, L.H., Vincent, M.A. and Barrett, E.J. (2007) Contraction stimulates nitric oxide independent microvascular recruitment and increases muscle insulin uptake. Diabetes **56**, 2194–2200 CrossRef PubMed

40 Park, S.Y., Cho, Y.R., Kim, H.J., Higashimori, T., Danton, C., Lee, M.K., Dey, A., Rothermel, B., Kim, Y.B., Kalinowski, A. et al. (2005) Unraveling the temporal pattern of diet-induced insulin resistance in individual organs and cardiac dysfunction in C57BL/6 mice. Diabetes **54**, 3530–3540 CrossRef PubMed

41 Turner, N., Kowalski, G.M., Leslie, S.J., Risis, S., Yang, C., Lee-Young, R.S., Babb, J.R., Meikle, P.J., Lancaster, G.I., Henstridge, D.C. et al. (2013) Distinct patterns of tissue-specific lipid accumulation during the induction of insulin resistance in mice by high-fat feeding. Diabetologia **56**, 1638–1648 CrossRef PubMed

42 Solinas, G. and Karin, M. (2010) JNK1 and IKKβ: molecular links between obesity and metabolic dysfunction. FASEB J. **24**, 2596–2611 CrossRef PubMed

43 Carvalho-Filho, M.A., Ropelle, E.R., Pauli, R.J., Cintra, D.E., Tsukumo, D.M., Silveira, L.R., Curi, R., Carvalheira, J.B., Velloso, L.A. and Saad, M.J. (2009) Aspirin attenuates insulin resistance in muscle of diet-induced obese rats by inhibiting inducible nitric oxide synthase production and S-nitrosylation of IRbeta/IRS-1 and Akt. Diabetologia **52**, 2425–2434 CrossRef PubMed

44 Fleischman, A., Shoelson, S.E., Bernier, R. and Goldfine, A.B. (2008) Salsalate improves glycemia and inflammatory parameters in obese young adults. Diabetes Care **31**, 289–294 CrossRef PubMed

45 Koska, J., Ortega, E., Bunt, J.C., Gasser, A., Impson, J., Hanson, R.L., Forbes, J., de Courten, B. and Krakoff, J. (2009) The effect of salsalate on insulin action and glucose tolerance in obese non-diabetic patients: results of a randomised double-blind placebo-controlled study. Diabetologia **52**, 385–393 CrossRef PubMed

46 Goldfine, A.B., Fonseca, V., Jablonski, K.A., Pyle, L., Staten, M.A. and Shoelson, S.E. (2010) The effects of salsalate on glycemic control in patients with type 2 diabetes: a randomized trial. Ann. Intern. Med. **152**, 346–357 CrossRef PubMed

47 Kim, Y.B., Nikoulina, S.E., Ciaraldi, T.P., Henry, R.R. and Kahn, B.B. (1999) Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. J. Clin. Invest. **104**, 733–741 CrossRef PubMed

Received 16 February 2015/21 July 2015; accepted 11 August 2015

Accepted Manuscript online 12 August 2015, doi: 10.1042/CS20150143

