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Postprandial Microvascular Blood Flow in Skeletal Muscle: Similarities and Disparities to the Hyperinsulinaemic Euglycaemic Clamp

Short title: Muscle blood flow

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Abstract

Skeletal muscle contributes to ~40% of total body mass and has numerous important mechanical and metabolic roles in the body. Skeletal muscle is a major site for glucose disposal following a meal. Consequently, skeletal muscle plays an important role in postprandial blood glucose homeostasis. Over the past number of decades, research has demonstrated that insulin has an important role in vasodilating the vasculature in skeletal muscle in response to an insulin infusion (hyperinsulinaemic euglycaemic clamp) or following the ingestion of a meal. This vascular action of insulin is pivotal for glucose disposal in skeletal muscle, as insulin-stimulated vasodilation increases the delivery of both glucose and insulin to the myocyte. Notably, in insulin-resistant states such as obesity and type 2 diabetes, this vascular response of insulin in skeletal muscle is significantly impaired. Whilst the majority of work in this field has focussed on the action of insulin alone on skeletal muscle microvascular blood flow and myocyte glucose metabolism, there is less understanding of how the consumption of a meal may affect skeletal muscle blood flow. This is in part due to complex variations in glucose and insulin dynamics that occurs postprandially - with changes in humoral concentrations of glucose, insulin, amino acids, gut and pancreatic peptides - compared to the hyperinsulinaemic euglycaemic clamp. This review will address the emerging body of evidence to suggest that postprandial blood flow responses in skeletal muscle may be a function of the nutritional composition of a meal.

Keywords: Insulin, postprandial, microvasculature, skeletal muscle, glycaemia

1. Haemodynamic Actions of Insulin: a Brief History

The first study to report on the vasodilatory actions of insulin in skeletal muscle was from the 1930's where bolus injections of insulin markedly increased limb blood flow [1]. However, it was uncertain at that time whether the effect was due to insulin *per se* or other counter-regulatory hormones (e.g. adrenaline) responding to the low blood glucose caused by the high dose of insulin. It was not until the 1990s where use of the hyperinsulinaemic euglycaemic clamp technique confirmed that insulin was indeed acting as a vasodilator in skeletal muscle [2-6]. The clamp technique involves a constant intravenous infusion of insulin to induce sustained hyperinsulinemia, while glucose is simultaneously infused at a variable rate to maintain euglycaemia. Maintaining euglycaemia offers important physiological advantages over other techniques, as the effect of insulin can be dissociated from changes in blood glucose levels or other counter-regulatory hormones. Using this technique Baron and colleagues advanced the field by demonstrating that insulin increases bulk skeletal muscle blood flow (or total muscle blood flow) in a dose-dependent fashion, which served to enhance insulin and glucose delivery (and uptake) into the muscle [3]. This vascular effect of insulin to stimulate limb blood flow was confirmed by others in both humans [7-9] and animals [10,11].

Early clamp work was criticised due to the supraphysiological insulin concentrations and lengthy exposure times used, which did not follow the dose and time course typically observed postprandially [12-14]. Furthermore, others contested the link between increased bulk blood flow and muscle glucose disposal by demonstrating that insulin-dependent increases in muscle glucose uptake occurred prior to changes in bulk blood flow [15,16]. Importantly, many studies using physiologically relevant concentrations of insulin (increases similar to those observed following a meal) showed no change in bulk flow following insulin infusion despite marked increases in muscle glucose disposal [17,18]. There were many factors proposed to explain the

'insulin flow controversy' including the dose and duration of the insulin infusion, the sensitivity of the technique used to measure blood flow, and individual variability between participants (e.g. the amount of muscle and the extent of capillarisation) [19]. However, one important aspect that was largely ignored until the early 2000's was the vascular site at which blood flow was being measured. There is now a growing body of literature to suggest that the regulation of blood flow at the microvascular level may be more important for nutrient and hormone delivery to the myocyte, and may explain temporal dissociations between bulk muscle blood flow and muscle glucose disposal [20,21].

2. Insulin Stimulated Skeletal Muscle Microvascular Blood Flow

The microvasculature (also known as capillaries) are the smallest blood vessels in the body and are the site of nutrient exchange between the blood and the tissue. While total muscle blood flow is primarily regulated by first to third order arterioles, the control of flow through the microvasculature is thought to occur further down the vascular tree between the third to fifth order arterioles [22,23]. The contraction and dilation of these upstream arterioles at different time intervals directs flow through different capillaries, in a process known as flowmotion. This process results in variable, oscillating flow through the capillary bed suggesting that under basal conditions not all capillaries are perfused at a given time [24,25]. During periods of metabolic demand such as exercise [26] or in the postprandial state [27-29], the number of capillaries that are actively perfused increases which facilitates nutrient and hormone delivery to the myocyte. The microvasculature can increase perfusion of the muscle independently of changes in bulk blood flow to the limb [30], which helps explain some of the 'insulin flow controversy' detailed above. Separating microvascular flow measurements from those of bulk flow is complex, but possible due to development of novel techniques (discussed below), each with its own advantages and limitations.

There are several techniques commonly used to measure microvascular blood flow in humans and animal models. Laser Doppler Flowmetry (LDF) is one such technique, widely used to measure skin and subcutaneous adipose tissue blood flow in humans [31,32] and muscle blood flow in rodents [33]. The LDF technique has the advantage of being non-invasive, simple to perform and data output can provide information regarding the underlying frequencies that contribute to flow distribution within the microvasculature. However, this technique has limited tissue penetration and therefore the laser is only able to sample flow in a small volume of tissue. While this technique can measure muscle microvascular blood flow in rodents (due to the thin layer of skin and subcutaneous adipose tissue) it has restricted application in humans as it can only measure superficial blood flow in the skin which does not reflect blood flow in the underlying skeletal muscle [34]. In addition, this technique measures red blood cell velocity which does not always reflect increased tissue perfusion [35].

Another approach to measuring muscle microvascular perfusion involves a biochemical method known as the 1-methylxanthine technique [36]. This technique involves infusing 1-methylxanthine systemically while monitoring the rate of 1-methylxanthine disappearance across a muscle bed. 1-methylxanthine is oxidised to 1-methylurate by capillary bound xanthine oxidase, and thus the decay in 1-methylxanthine is an indicator of microvascular blood flow. While this technique has successfully been used in rodents to measure changes in microvascular perfusion [36,37], its use is limited as it cannot be used in humans due to low xanthine oxidase activity in the microcirculation of human skeletal muscle. Given the limitations of LDF and 1-methylxanthine techniques in humans, the contrast enhanced ultrasound (CEU) was adapted to assess microvascular blood volume and filling rate in real-time in skeletal muscle of humans and animals [38,39]. This technique combines ultrasound imaging with the infusion of a contrast agent (microbubbles). Microbubbles interact with

ultrasound to produce an acoustic signal, reflecting the volume of perfused vessels, which can be imaged in real-time and recorded for subsequent analysis [39].

The development and application of these techniques over the past 20 years has contributed greatly to our understanding of microvascular blood flow and has helped to validate and expand upon the early observations of Abramson and colleagues in the 1930's [1]. These techniques have been applied to assess the mechanism, timing and responsiveness of microvascular flow to insulin (and other dilators) in a number of models. Many of the observations have been undertaken during the hyperinsulinaemic euglycaemic clamp technique in animals and humans [40,41]. In addition to stimulating bulk blood flow to skeletal muscle, insulin also increases skeletal muscle microvascular blood flow [30]. This effect is similar to skeletal muscle contraction (although the magnitude of the effect is greater with contraction) and this is observed in both animals and humans [30,42-45]. The microvascular action of insulin occurs independently of changes in bulk blood flow to skeletal muscle [45,46] and precedes stimulation of the insulin signalling cascade that leads to glucose uptake by the myocytes [40]. Insulin-stimulated skeletal muscle microvascular blood flow is a sensitive process where increases are observed in rats at low physiological concentrations (1 mU/min/kg) [47] and very quickly, exerting its full effect by 15-20 mins [40,45]. Insulin-stimulated microvascular blood flow in skeletal muscle of rats is slow to reverse- taking up to 30 minutes after insulin has returned to basal levels, indicating the process remains activated after insulin is no longer present [47].

The importance of microvascular blood flow on muscle metabolism is supported by considerable evidence showing that they are intimately linked [40]. Indeed this concept is well-accepted for contraction-induced metabolism and microvascular (and bulk) blood flow [48]. In the first instance, it has been demonstrated that inhibiting insulin-mediated microvascular flow

can induce a state of acute insulin resistance in the underlying skeletal muscle [41,49,50]. Importantly, these experiments have demonstrated that the microvascular action of insulin contributes to ~40% of total insulin-mediated glucose disposal in skeletal muscle in clamp conditions [41,51]. Secondly, chronic states of insulin resistance are accompanied by an impaired ability for the microvasculature to respond to insulin, both in the presence of skeletal myocyte insulin resistance and in its absence.

3. Acute Microvascular Insulin Resistance

Vascular insulin resistance is a term that refers to a blunted vasodilatory response to insulin at an endothelial level [52]. Several vasoconstrictors have been shown to oppose insulin-mediated vasodilatory actions and cause an acute state of insulin resistance. The acute infusion of endothelin-1 (ET-1) [53], α-methyl serotonin [54] or angiotensin II [55] each block insulin-stimulated microvascular blood flow which is coupled to a reduction in muscle glucose uptake by 40-50%. Notably, a number of studies have investigated the impact of acute elevation of circulating free fatty acids (FFA's) and pro-inflammatory cytokines (TNFα) on muscle microvascular function, both of which blunt insulin-stimulated microvascular blood flow and muscle glucose disposal in healthy rats [56] and human subjects [57]. Taken together, these findings highlight the susceptibility of the microvasculature to an acute insult, which can consequently impede insulin's microvascular-linked metabolic actions in skeletal muscle.

4. Chronic Microvascular Insulin Resistance

There is a strong school of thought that in insulin resistant states, the key impairment lies within the myocyte [58-61]. However, there is a strong evidence that in insulin resistant states, the vasculature (including the microvasculature) also becomes insulin resistant [19,62]. For example, several high-fat fed animal models - which develop insulin resistance - display marked

impairments in insulin-stimulated muscle microvascular blood flow [50,63-66]. This has been noted in mice [50], rats [63-65] and non-human primates [66]. Of significance is the finding that microvascular insulin resistance has been observed as early as 3 days into high-fat feeding [65] demonstrating the sensitivity of high-fat diets on the microvasculature. Similar effects are seen in the obese Zucker rat, homozygous for the leptin receptor mutation [67], and in obese humans [43]. These findings emphasise the contribution of chronic obesity to microvascular dysfunction and subsequent insulin resistance, particularly in skeletal muscle.

The cross-talk between the microcirculation and the myocyte is important to consider because if one of these tissues become resistant to the actions of insulin, this results in skeletal muscle insulin resistance *in vivo*. When both of these tissues (microvasculature and myocyte) become resistant to the actions of insulin, this leads to a more severe form of insulin resistance [67-70] . This is due to the additive effect of impaired delivery of insulin and glucose to the interstitial space (microvascular insulin resistance) and the failure of the myocyte to take up the glucose (myocyte insulin resistance). There is a growing body of evidence to suggest that the microvasculature can become insulin resistant well before myocyte insulin resistance. Knockout animal models have also facilitated our understanding of microvascular insulin resistance. Kubota and colleagues were the first group to develop an endothelial insulin receptor subtrate-2 (IRS-2; the major isoform expressed on endothelial cells) knockout mouse model [50]. These mice have impaired insulin-induced endothelial nitric oxide synthase (eNOS) phosphorylation, impaired microvascular responses to insulin and consequently reduced skeletal muscle glucose disposal [50]. This animal model had normal insulin-mediated glucose uptake when assessed by *ex vivo* muscle incubation, highlighting that the impaired glucose disposal *in vivo* was related to microvascular and not metabolic insulin resistance. Moreover, Bonner et al. developed a vascular endothelial growth factor (VEGF) knockout mouse,

deficient of the VEGF gene in skeletal and cardiac muscle [71]. This mouse model displayed significant skeletal (and cardiac) muscle capillary rarefaction, coupled with diminished muscle insulin-mediated glucose uptake *in vivo*. However, insulin-mediated skeletal muscle glucose uptake was not blunted in *ex vivo* muscle incubations, where the myocyte is in direct contact with insulin and glucose (i.e. no vascular delivery) demonstrating that the myocyte in this animal model is not insulin resistant. Taken together, these models lead to suggest that reduced muscle glucose disposal observed *in vivo* is likely to have microvascular origins.

Additional evidence that vascular insulin resistance precedes that of the myocyte is provided via dietary interventions in rodents that induce skeletal muscle microvascular insulin resistance. For example, a two-fold increase in dietary fat (4.8% fat wt. /wt. to 9.0% fat wt. /wt.) for 4 weeks blunts insulin-stimulated microvascular blood flow and glucose disposal *in vivo*, but not in the fully vasodilated *in situ* constant flow perfused rat hindlimb preparation [70]. Similarly, 4 weeks of high-sodium intake (0.31% wt./wt. NaCl to 8.0% wt./wt.) also produced a similar microvascular insulin resistant model, in the absence of myocyte or liver insulin resistance [72]. Taken together, this work suggests that microvascular insulin resistance is likely to be an early hallmark of broader metabolic insulin resistance and emphasises the importance of microvascular function in skeletal muscle glucose disposal. The vast majority of work focussed on vascular insulin resistance has investigated large artery and endothelial function in individuals with type 2 diabetes, insulin resistance and obesity [73,74]. Whilst the literature suggests that large artery insulin-mediated vasodilation is impaired in such individuals [3], its impact on muscle insulin resistance is not entirely understood. Microvascular insulin resistance may contribute to the development of myocyte insulin resistance [70-72], and the mechanisms of (micro)vascular insulin resistance have therefore become an area of increasing interest.

However, before we can speculate on such mechanisms, it is important to review what is known about the mechanism of insulin-induced microvascular flow in the healthy state.

5. Mechanisms of Insulin-Stimulated Microvascular Blood Flow

There is now a general consensus that insulin stimulates bulk blood flow and microvascular blood flow via the stimulation of nitric oxide synthase (NOS), producing the potent vasodilator nitric oxide (NO) from the vascular endothelium (outlined in Figure 1) [75,76]. Cell culture work demonstrates that the production of NO occurs via an insulin signalling pathway in the vascular endothelium, which also shares a number of steps in common with the insulin signalling pathway in myocytes [75,77]. In muscle, the activation of this pathway results in GLUT4 translocation to the membrane facilitating glucose uptake, whereas in the vascular endothelium this results in phosphorylation and activation of eNOS, resulting in NO production [77]. Quon and colleagues have characterised the insulin signalling cascade in vascular endothelial cells confirming that insulin acts via the insulin receptor/IRS-1,PI3K/AKT/eNOS pathway resulting in NO production [78,79]. NO then diffuses into the adjacent vascular smooth muscle cells, leading to vasodilation of pre-capillary arterioles resulting in increased microvascular blood flow [80].

This is further supported by a number of animal studies showing that when a NOS-specific inhibitor (e.g. N^G- nitro- L- arginine methyl ester; L- NAME) is infused intravenously, this blocks most, if not all, insulin-dependent increases in microvascular blood flow [40,41,81]. We have demonstrated that blocking insulin-stimulated muscle microvascular blood flow with L-NAME impairs skeletal muscle glucose disposal by up to 40% [41,51]. These studies have used the hyperinsulinaemic euglycaemic clamp technique where insulin is infused systemically, raising the question as to whether insulin acts locally in the muscle vascular bed, or centrally (activating the central nervous system) due to insulin's ability to cross the blood brain barrier.

Local infusion of insulin into the forearm of healthy individuals, at a concentration that causes no detectable spill-over into the systemic circulation, augments microvascular blood flow which is paralleled by increased muscle glucose uptake [44]. Furthermore, we have also demonstrated that central (intracerebroventricular) insulin administration in healthy rodents does not lead to haemodynamic or metabolic changes in skeletal muscle [82]. Interestingly, when L-NAME is infused locally into one leg of a rat undergoing a systemic hyperinsulinaemic euglycaemic clamp, the microvascular actions of insulin are completely lost [48]. Work undertaken using an IRS-2 knockout mouse model supports the notion that NO production is endothelium-dependent. Kubota and colleagues [50] have shown that deletion of endothelial specific IRS-2 results in reduced insulin-dependent eNOS phosphorylation, NO production and blunted skeletal muscle glucose disposal. Taken together, these studies suggest that insulin's microvascular actions in skeletal muscle are mediated at the local muscle level rather than by central NOS-mediated mechanisms.

In addition to the well-documented vasodilatory actions of insulin on the vascular endothelium [75], insulin also stimulates the production of a potent vasoconstrictor [83]. ET-1 is produced from endothelial cells in response to an alternate insulin activated pathway (MAPK; outlined in figure 1), and has opposing actions to the vasodilator NO [84]. The precise regulation of ET-1 is now well known, however it is thought that a tightly coupled balance between NO and ET-1 production is vital for optimal vascular function and resultant flow regulation, with NO being the dominant vasomodulator stimulated by insulin under healthy circumstances [85]. In support of this, acute infusion of ET-1 blocks both insulin-mediated microvascular blood flow and glucose disposal in healthy rats *in vivo* [53]. Insulin-mediated large artery vasodilation is augmented by ET-1 receptor blockade [86,87]. In addition, patients with type 2 diabetes also exhibit a shift in the NO and ET-1 balance, leading to increased vascular tone and derecruitment

of capillaries [88]. Thus, insulin causes both vasodilation (NO) and vasoconstriction (ET-1), and it is the delicate balance between these two vasomodulators that contributes to the regulation of microvascular blood flow in skeletal muscle.

Evidence is now emerging to suggest that other endothelial-derived factors may be involved in augmenting skeletal muscle microvascular blood flow [89]. Molecules formed from arachidonic acid- epoxyeicosatrienoic acids - can stimulate microvascular blood flow on their own, and when their production is blunted, insulin's ability to increase microvascular blood flow is also blocked [89]. The same research group have also reported epoxyeicosatrienoic acids to be vasoconstrictive, rather than vasodilatory, in insulin resistant states [90]. While interesting, it should be noted that these studies were performed using an intravenous glucose tolerance test rather than a hyperinsulinaemic euglycaemic clamp and requires further investigation.

6. Postprandial Effects on Skeletal Muscle Microvascular Blood Flow

The vast majority of our understanding of insulin-stimulated microvascular blood flow has developed from work using the hyperinsulinaemic euglycaemic clamp technique. Whilst this technique enables precise investigation of microvascular responses to an insulin infusion, it does not reflect the postprandial state in which multiple physiological responses occur simultaneously [26]. The clamp technique is conducted under a controlled setting, and therefore does not take into account the dynamic changes in blood glucose, amino acids and resultant insulin levels observed following a meal [91]. Moreover, the clamp technique bypasses the release of gut-derived factors known as incretins, namely glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), of which have been demonstrated to have notable effects on peripheral metabolism [92]. Research performed over the past decade has suggested a strong positive association between GLP-1 and microvascular blood flow in both animals and humans

[93-96], and therefore warrants further consideration regarding its involvement in the regulation of blood glucose in the postprandial state. Furthermore, GLP-1 is secreted in a dose-dependent fashion to increasing amounts of dietary carbohydrate [97], suggesting that the nutrition composition of a meal is an important consideration. Therefore, it is imperative to characterise skeletal muscle microvascular function in response to meals, and more specifically meals of varying nutritional composition.

7. Effects of Mixed Meal Challenges on Skeletal Muscle Microvascular Blood Flow: The Early Studies

Our group, along with our collaborators at the University of Virginia, were the first to report the effect of a mixed meal challenge (blend of lipid, carbohydrate and protein) on muscle microvascular blood flow [26,98]. We demonstrated that the mixed meal was a stimulus for microvascular blood flow within the skeletal muscle of healthy individuals [43,98]. Importantly, the microvascular response was comparable to that observed during a hyperinsulinaemic euglycaemic clamp [43,57]. It was proposed that this microvascular blood flow response was likely due to meal-induced hyperinsulinemia. Importantly, people with insulin resistance exhibited a marked reduction in meal-related microvascular blood flow [98], which mirrored the microvascular responses observed in the hyperinsulinaemic euglycaemic clamp [43]. Interestingly, multiple studies performed in the last decade have shown inconsistent microvascular blood flow responses to a mixed meal when compared to a hyperinsulinaemic euglycaemic clamp [99-101]. The reason behind these divergent responses are not known, however in this review we propose that the nutritional composition of the mixed meal may be a key determinant of the postprandial microvascular response.

8. Mixed Meal Challenges and Microvascular Blood Flow: A More Complex Relationship

Few studies have assessed the impact of meal ingestion on muscle microvascular blood flow in animal models. Most investigators who have assessed muscle blood flow responses to a meal in humans have focused on total limb blood flow [102,103]. As such, only a few have measured skeletal muscle specific microvascular blood flow [26,57,98]. It has been widely reported that skeletal muscle microvascular blood flow is stimulated during a hyperinsulinaemic euglycaemic clamp in healthy people, and that this response is lost as a result of insulin resistance or type 2 diabetes. However, measurements of meal-related microvascular blood flow responses in health and metabolic disease have produced mixed results (summarised in Table 1).

Liu et al reported that a mixed meal challenge (68% calories from carbohydrate, 17% protein and 15% fat, 9-fold increase in plasma insulin), stimulated muscle microvascular blood flow in healthy people [57]. Others have also shown similar microvascular blood flow responses in healthy people following an essential amino acid meal (15g mixed essential amino acids, 3-fold increase in plasma insulin), and this effect was blunted in older participants [104,105]. Thus, it appears at first glance that meals that stimulate insulin release in healthy people also stimulate muscle microvascular blood flow (whether a mixed meal or as an amino acid single nutrient). However, we have recently examined the relationship between pure glucose ingestion on skeletal muscle microvascular function. The ingestion of a 50g oral glucose challenge showed no increases in skeletal muscle microvascular blood flow of healthy subjects despite a rise in plasma insulin, suggesting microvascular impairment [106]. In contrast, a liquid mixed meal matched for pancreatic insulin secretion, enhanced microvascular blood flow in the same participants [106]. These findings are also supported by Tobin and colleagues who demonstrated loss of microvascular blood flow in forearm skeletal muscle of healthy individuals following the ingestion of a 75g glucose load [107]. Moreover, cell culture work using rat adipocytes has

shown that when cells are exposed to hyperinsulinemia and hyperglycaemia concomitantly, a marked reduction in insulin's binding capacity and downregulation of insulin signalling proteins is noted [108]. We propose that the extent of the hyperglycaemia may play a role in the microvascular impairment observed in both our study [106] and those performed by others [107]. It is possible that when blood glucose levels rise above a critical threshold in healthy people, this results in a reduction in meal-related microvascular blood flow responses in skeletal muscle. Mertz and colleagues investigated skeletal muscle microvascular responses to a high protein and carbohydrate mixed meal (containing 20g whey protein hydrolysate and 80g maltodextrin) [100]. Given the high maltodextrin content of the meal, it is perhaps not surprising that microvascular blood flow changes were absent postprandially. Given this study did not report blood glucose or insulin excursions following the meal, it can only be speculated that this meal had a high glycaemic index, which may explain the absence of microvascular blood flow responses in this study.

Others have determined that insulin switches from dilation to constriction of pre-capillary arterioles in the presence of hyperglycaemia in certain animal models [11]. For example, it has been demonstrated that short-term hyperglycaemia (for 24hrs) in cell culture studies prominently impairs insulin-mediated eNOS activation [109]. Kolka and colleagues have shown that modest hyperglycaemia in dogs impairs insulin access to the skeletal muscle interstitium [110], resulting in reduced insulin transport to the myocyte surface and thus, blunted insulin-mediated muscle glucose disposal [110]. Whether this is linked to an impairment in microvascular blood flow is unknown, though plausible.

The content of fat in the meal may also be an important determinant for meal-related microvascular responses. Jahn et al have demonstrated that a single high fat meal (60% calories

from fat, 26% protein and 14% carbohydrate, ~8-fold increase in plasma insulin), does not stimulate microvascular blood flow in skeletal muscle of healthy people [99]. Importantly after the high fat meal, the participants were subjected to a hyperinsulinaemic euglycaemic clamp in which the effect of insulin to stimulate microvascular blood flow was found to be blocked [99].

In summary, the macronutrient composition of a meal may dictate skeletal muscle microvascular responses postprandially and could account for some of the disparities seen in the responses of healthy individuals. More specifically, the studies detailed above suggest that the fat and carbohydrate content of a mixed meal may be crucial in governing the microvascular response. However, given the array of physiological responses that occur postprandially, more research is required to understand the mechanisms behind this.

9. Gaps in the Literature

Over the century, our understanding of the mechanisms governing skeletal muscle microvascular recruitment in response to an insulin infusion has been the topic of much research [78,79]. However, less is known regarding the mechanisms that regulate microvascular blood flow following the ingestion of a meal. Whilst it has been proposed that augmented microvascular blood flow can be attributed to a surge in insulin postprandially [104], additional mechanisms such as gut-derived factors, some of which have been shown to stimulate microvascular blood flow, cannot be overlooked. Our current understanding of the regulation of postprandial microvascular blood flow in healthy subjects is particularly limited, emphasising the need for further research.

Firstly, there are still considerable gaps in our understanding of the contribution of skeletal muscle microvascular action to skeletal muscle glucose disposal in the postprandial state.

Whilst this relationship has been explored using the hyperinsulinaemic euglycaemic clamp technique, our emerging understanding of the postprandial state has highlighted the disparities between the clamp technique and meal ingestion, suggesting that this relationship and consequently the mechanisms governing microvascular flow and glucose disposal may differ. Secondly, there are notable inconsistencies seen in skeletal muscle microvascular responses to meals containing varying fat, glucose and protein constituents. This suggests that more work is required to understand the muscle microvascular blood flow responses to different amounts of macronutrients (particularly fat and carbohydrate) consumed alone and in mixed meals. Similarly, there is little known regarding the mechanisms behind blunted and impaired microvascular blood flow observed following the ingestion of high fat, high carbohydrate mixed meals, and glucose alone [99-101,106,107].

Thirdly, the literature suggests that a blunted microvascular blood flow response is coupled with a reduction in skeletal muscle glucose disposal [49,55]. However, it is not known whether insulin or glucose delivery to the myocyte is the rate-limiting step. Kolka and colleagues have proposed that insulin delivery to the myocyte is significantly impaired in the presence of hyperglycaemia [110], however whether this is in fact rate-limiting and if it results from blunted microvascular blood flow is not firmly established.

10. Conclusions

The haemodynamic actions of insulin have been of interest to many since as early as the 1930's. It is thought that whilst insulin typically acts to increase large artery blood flow in human limbs [1,3] it is the microvascular actions of insulin which are more important in facilitating the delivery of insulin and glucose to the skeletal muscle [20,21], thus facilitating glucose disposal. The majority of work performed in this area has utilised the hyperinsulinaemic euglycaemic clamp

technique, which enables the investigation of skeletal muscle microvascular responses to an insulin infusion, however this technique bypasses a number of physiological responses that occur following the ingestion of a meal. Interestingly, there are inconsistencies observed in the microvascular responses to mixed meals as well as single macronutrients, with evidence to suggest that the fat and carbohydrate content of a meal could dictate this response [99,100]. A small body of work has focussed specifically on the relationship between acute glycaemia and microvascular function [106,107], with evidence to suggest that elevation of plasma glucose above a critical point could be detrimental to microvascular action [106]. Considerable gaps in our understanding of this relationship remain, with little known regarding other factors such as gut-derived hormones (or other factors) that may be playing a role in microvascular regulation. The implications of hyperglycaemia-induced microvascular dysfunction on skeletal muscle glucose disposal is still an important notion that requires investigation. Furthering our understanding of this seemingly complex relationship has the potential to influence type 2 diabetes therapeutic strategies, as well as accentuates the effects of acute microvascular dysfunction in healthy individuals. These are areas that we are actively investigating.

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Figure Legends

Figure 1. Insulin signalling pathways within skeletal muscle arteriole endothelial cells. Illustration of branching of the vascular network within skeletal muscle, with larger 2º arterioles branching off into 3º, 4º, 5º order arterioles leading to capillaries. Within arteriole endothelial cells, insulin activates two different signalling pathways. When insulin binds to the insulin receptor activating IRS2, PI3K, Akt and eNOS, this produces the vasodilator NO that enables vasorelaxation of the vascular smooth muscle. When insulin activates the alternate MAPK signalling pathway within arteriole endothelial cells, this produces the vasoconstrictor ET-1 which causes vasoconstriction of the surrounding vascular smooth muscle.

Table 1. Key findings on the effect of insulin infusion (hyperinsulinaemic euglycaemic clamp) and meal consumption on microvascular responses in skeletal muscle assessed by contrast-enhanced ultrasound imaging. Data expressed as means ± standard deviation. ↑= increase, ↔= no change; ↓ = decrease.

Population	Intervention	Microvascular Response	Comments	Reference
Healthy Subjects				
Healthy -Age: 30±6yrs -BMI: 24±3kg/m^2	Hyperinsulinaemic euglycaemic clamp	↑	Insulin infusion: 0.05mU/kg/min x4hrs	Coggins et.al. [44]
Healthy -Age: 37±10yrs -BMI: 23±3kg/m^2	Hyperinsulinaemic euglycaemic clamp	↑	Insulin infusion: 1mU/kg/min x2hrs	Clerk et.al. [43]
Healthy -Age: 24±4yrs -BMI: 23±4kg/m^2	Hyperinsulinaemic euglycaemic clamp	↑	Insulin infusion: 1mU/kg/min x2hrs	Eggleston et.al. [45]
Healthy -Age: 23±5yrs -BMI: 21.8±2.4kg/m^2	Hyperinsulinaemic euglycaemic clamp	↑	Insulin infusion: 2mU/kg/min x10min 1mU/kg/min x 110min	Liu et.al. [57]
Healthy -Age: 31±11yrs -BMI: 25.7±5.6kg/m^2	Hyperinsulinaemic euglycaemic clamp	↑	Insulin infusion: 1mU/kg/min x2.5hrs	Meijer et.al. [111]

Healthy -Age: 42yrs -BMI: 22.4 kg/m^2	Hyperinsulinaemic euglycaemic clamp	↑	Insulin infusion: 40mU/m^2/min x2hrs	Meijer et.al. [112]
Healthy -Age: 43±12yrs -BMI: 23±2kg/m^2	Hyperinsulinaemic euglycaemic clamp	↑	Insulin infusion: 2mU/kg/min x10min 1mU/kg/min x 110min	Jahn et.al. [99]
Healthy -Age: 25±4yrs -BMI: 23.2±0.7kg/m^2	Hyperinsulinaemic euglycaemic clamp	↑	Insulin infusion: 1.4mU/kg/min x195mins	Sjøberg et.al. [48]
Healthy -Age: 29±9yrs -BMI: 23±4kg/m^2	Hyperinsulinaemic euglycaemic clamp	↑	Meal: 68% carbohydrate, 17% protein and 15% fat. Peak blood glucose: 6.7±0.2mM	Vincent et.al. [26]
Healthy -Age: 41±12yrs -BMI: 22.4±2.5kg/m^2	Mixed meal	↑	Meal: 68% carbohydrate, 17% protein and 15% fat. Peak blood glucose: ~6.7mM	Keske et.al. [98]
Healthy -Age:24±5yrs -BMI: 22.6±2.2kg/m^2	Mixed meal	↑	Meal: 68% carbohydrate, 17% protein and 15% fat. Peak blood glucose: 7±0.4mM	Liu et.al. [57]
Healthy -Age:46±12yrs -BMI: 25.4±2.7kg/m^2	Mixed meal	↑	Meal: 55% carbohydrate, 29% protein and 14% fat. Peak blood glucose: ~5.9mM	Russell et.al. [106]
Healthy -Age:20±2yrs -BMI: 22.5±2.7kg/m^2	Amino acids	↑	Meal: 15g oral mixed essential amino acids. Peak blood glucose: not reported.	Mitchell et.al. [104]
Healthy -Age:20±1yrs -BMI: 22.9±2kg/m^2	Amino acids	↑	Meal: 15g essential amino acids. Peak blood glucose: not reported.	Mitchell et.al. [105]

Healthy -Age:24±3yrs -BMI: 21.6±1.6kg/m^2	Mixed meal	↔	Meal: 81% Maltodextrin (sugar) and 19% protein. Peak blood glucose: not reported.	Mertz et.al. [100]
Healthy -Age:43±12yrs -BMI: 23±2kg/m^2	Mixed meal	↔	Meal: 60% fat, 26% protein and 14% carbohydrate. Peak blood glucose: ~6.2mM	Jahn et.al. [99]
Healthy -Age:44±9yrs -BMI: Not reported	Glucose	↔	Meal: 75g glucose Peak blood glucose: Not reported	Tobin et.al. [107]
Healthy -Age:46±12yrs -BMI: 25.4±2.7kg/m^2	Glucose	↓	Meal: 50g glucose Peak blood glucose: ~8.1mM	Russell et.al. [106]
Healthy -Age:41±15yrs -BMI: 24.7±3.0kg/m^2	Glucose	↔	Meal: 50g glucose Peak blood glucose: 7.7±0.3mM	Russell and Roberts-Thomson et.al. (Unpublished)
Metabolically Impaired Subjects				
Overweight -Age:43±6yrs -BMI: 34±3kg/m^2	Hyperinsulinaemic euglycaemic clamp	↔	Insulin infusion: 1mU/kg/min x2hrs	Clerk et.al. [43]
Overweight -Age:41yrs -BMI: 33kg/m^2	Hyperinsulinaemic euglycaemic clamp	↔	Insulin infusion: 40mU/m^2/min x2hrs	Meijer et.al. [112]
Metabolic Syndrome -Age: 46±13yrs -BMI: 35±9kg/m^2	Hyperinsulinaemic euglycaemic clamp	↔	Insulin infusion: 2mU/Kg/min x10min 1mU/Kg/min x 110min	Jahn et.al. [99]

Type 2 Diabetes -Age: 55yrs -BMI: 33.1 kg/m^2 -Fasting glucose: 8.0mM	Hyperinsulinaemic euglycaemic clamp	↓	Insulin infusion: 40mU/m^2/min x2hrs	Emanuel et.al. [88]
Overweight -Age:42±8yrs -BMI: 33.7±2.8kg/m^2	Mixed meal	↔	Meal: 68% carbohydrate, 17% protein and 15% fat. Peak blood glucose: ~6.7mM	Keske et.al. [98]
Metabolic Syndrome -Age: 46±13yrs -BMI: 35±8.5kg/m^2	Mixed meal	↔	Meal: 60% fat, 26% protein and 14% carbohydrate. Peak blood glucose: ~7.1mM	Jahn et.al. [99]
Elderly -Age: 70±3yrs -BMI: 25.6±2.3kg/m^2	Amino acids	↔	Meal: 15g oral mixed essential amino acids. Peak blood glucose: not reported.	Mitchell et.al. [104]
Elderly -Age: 70±3yrs -BMI: 25.5±1.6kg/m^2	Amino acids	↔	Meal: 15g essential amino acids. Peak blood glucose: not reported.	Mitchell et.al. [105]
Type 2 Diabetes -Age: 58±11yrs -BMI: 31±9kg/m^2 -Fasting glucose: 7.8±0.7	Glucose	↔	Meal: 75g glucose Peak blood glucose: 15.5±1.2mM	Tobin et.al. [113]
Type 2 Diabetes -Age:55±6yrs -BMI: 33.1±5.2kg/m^2 -Fasting glucose: 11.2±1.5mM	Mixed meal	↔	Meal: 55% carbohydrate, 29% protein and 14% fat. Peak blood glucose: 12.3±1.3mM	Russell and Roberts-Thomson et.al. (Unpublished)
Type 2 Diabetes -Age:55±6yrs -BMI: 33.1±5.2kg/m^2 -Fasting glucose: 11.2±1.5mM	Glucose	↔	Meal: 50g glucose Peak blood glucose: 15.6±1.1mM	Russell and Roberts-Thomson et.al. (Unpublished)