***In vivo* differential effects of fasting, re-feeding, insulin and insulin stimulation time course on insulin signaling pathway components in peripheral insulin-sensitive tissues**

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Abstract

Background: Components of the insulin receptor signaling pathway are probably some of the best studied ones. Even though methods for studying these components are well established, the *in vivo* effects of different fasting regimens, and the time-course of insulin receptor phosphorylation and that of its downstream components in insulin-sensitive peripheral tissues have not been analyzed in detail. **Rationale:** When assessing insulin signalling, it may be beneficial to drive insulin levels as low as possible by performing an overnight fast before injecting a supra-physiological dose of insulin. Recent studies have shown however that 5 or 6 hour fast in mice is sufficient to assess physiological responses to insulin and/or glucose in glucose tolerance tests {{200 Andrikopoulos,S. 2008; }}, insulin tolerance tests {{2 Bence,K.K. 2006; 1 Delibegovic,M. 2007; 105 Delibegovic,M. 2009; }} and euglycemic hyperinsulinemic clamps {{201 Ayala,J.E. 2006; 202 Preitner,F. 2009; }}. Moreover, mice are nocturnal feeders, with ~70% of their daily caloric intake occurring during the dark cycle {{201 Ayala,J.E. 2006; }}, and their metabolic rate is much higher than humans. Therefore, an overnight fast is a comparatively long time compared with humans and more akin to starvation. Thus our aim was to assess insulin signalling from the insulin receptor to downstream targets PKB, GSK3 and S6 in muscle, liver and adipose tissue in 5 hour versus 16 hour fasted mice, and the time-course of this phosphorylation. We also assessed whether re-feeding was a more robust stimulus than insulin alone.

Introduction

One of the most investigated signaling pathways is the cascade of events triggered by insulin binding to its receptor as disruption in normal insulin signaling responses is associated with the pathogenesis of insulin resistance in type 2 diabetes and obesity. Insulin receptor (IR) comprises two extracellular α subunits and two transmembrane β subunits. Activation of IR leads to autophosphorylation of both β subunits on tyrosine residues, with the major sites being at Tyr-1158, Tyr-1162 and Tyr-1163 {{62 Salmeen,A. 2000; }}. Autophosphorylation of these three sites is required for its maximal activation {{171 White,M.F. 1988; }}. Once tyrosine kinase activity of the IR β subunit is activated, insulin signaling pathways diverge, with one pathway proceeding through the insulin receptor substrates IRS-1 and IRS-2 and activation of phosphatidylinositol 3-kinase (PI3K), with subsequent activation of downstream targets such as Akt (PKB), with another pathway proceeding through Grb2/Sos and ras, leading to activation of MAP kinase isoforms ERK1 and ERK2 {{172 Cusi,K. 2000; }}. Insulin also activates other

protein kinases belonging to the same subfamily of protein kinase as Akt/PKB. These include p70 ribosomal S6 kinase (S6K), as well as the serum and glucocorticoid induced protein kinase (SGK). The activation of S6K and SGK by insulin is dependent on PI 3-kinase, and these enzymes possess a similar activation loop and hydrophobic motif to PKB {{4 Lizcano,J.M. 2002; }}. Details of the insulin receptor signaling have mostly been revealed by the use of variety of *in vitro* techniques, although the definitive answers as to the physiological role(s) of the signaling components has come from *in vivo* knockout mouse models (for Review see {{9 Taniguchi,C.M. 2006; }}).

In order to study and understand what goes wrong under pathological conditions such as insulin resistance and type 2 diabetes *in vivo*, we first need to have the right experimental conditions and know the time course of insulin's effects on its signaling components in different insulin-sensitive tissues in normal, healthy mice. Most *in vivo* studies investigating insulin signaling pathways in mice use C57Bl6 mouse model with an overnight, 16-hour fast, before a terminal insulin signaling procedure {{105 Delibegovic,M. 2009; 1 Delibegovic,M. 2007; 2 Bence,K.K. 2006; 108 Klamann,L.D. 2000; }}; however, this prolonged period of fasting induces many physiological changes, leads to a dramatic weight loss and depletes hepatic glycogen stores {{170 Irimia,J.M. 2010; }}. In fact, many knockout mouse models are not able to withstand this shock of overnight fasting, such as for example, mice lacking glutathione peroxidase 1 (Gpx1-/-) {{169 Loh,K. 2009; }} whereby a 4-hour fast had to be performed for the insulin signaling experiments; for other reasons perhaps, some studies also use a shorter 6-hour fast, for example, experiments conducted on adipose triglyceride lipase (ATGL) deficient mice {{173 Kienesberger,P.C. 2009; }}. In addition, most commonly, only one time-point of insulin stimulation is used *in vivo*; with the most common one being a 10-minute intraperitoneal injection, to investigate signaling events in all the insulin-sensitive tissues, such as skeletal muscle, liver and adipose tissue.

In order to determine the most appropriate experimental conditions for insulin signaling studies, we examined cellular signaling events in peripheral insulin-sensitive tissues following variation in fasting period, variation in stimulus or variation in stimulus period, thereby:

- (1) a short-term (5 hour) food withdrawal or a prolonged (16 hour) fast,
- (2) insulin injection or re-feeding,
- (3) a time course up to 30 minutes following insulin injection.

We present here that food withdrawal from mice for a short period of time of 5 hours results in a similar fasting, insulin and re-feeding response in phosphorylation events of the insulin receptor (IR), insulin receptor substrate 1 (IRS1), Akt/PKB, Erk1/2 and S6 as in mice fasted for a prolonged period of 16 hours in all tissues examined. We also investigated the *in vivo* time-course of phosphorylation events in peripheral insulin sensitive tissues following intraperitoneal insulin injections for 0, 2, 5, 10, 15 and 30 minutes. These revealed differences in time course of phosphorylation events, with muscle and liver insulin signaling components peaking at 10-15 minutes post insulin injection and remaining sustained post-IR phosphorylation, whilst adipose tissue insulin signaling peaked and returned towards basal levels after 15 and 30 minutes, with different time courses observed for individual signaling molecules. Our study determines that a short food withdrawal from mice, for a period of 5 hours, results in a similar insulin-stimulated response in phosphorylation events as the long overnight fast presenting a

more physiological experimental set up. We also demonstrate that *in vivo*, insulin stimulated phosphorylation of its signaling components is different between different peripheral tissues, and depending on the tissue(s) and protein(s) of interest, an appropriate time-course should be chosen.

Results

Effects of fasting, insulin and re-feeding on circulating glucose and insulin levels

As previously reported, overnight fasting results in greater loss of total body, lean, and fat masses and hepatic glycogen but results in enhanced insulin sensitivity compared with 5 hour fasting [201 Ayala, J.E. 2006;]. In our studies, basal insulin levels were the same in mice fasted for either 5 hours or 16 hours (Figure 1A); however, basal glucose levels were 2-fold higher in 5 hour fasted mice compared to 16 hour fasted mice (Figure 1B). Following supra-physiological dose of insulin by intraperitoneal injection, circulating insulin levels rose similarly (~42-fold) and blood glucose fell about 36% in both 5 hour fasted and 16 hour fasted mice compared to their respective saline treated controls (Figure 1A and 1B). In contrast, re-feeding for a period of 45 minutes, led to a more modest, physiological increase in insulin levels (~4-fold) in both 5 hour fasted and 16 hour fasted mice compared to their respective saline treated controls (Figure 1A). In addition, blood glucose levels rose 2-fold in 16 hour fasted mice but did not change significantly in 5 hour fasted mice (Figure 1B).

Effects of fasting, insulin and re-feeding on phosphorylation events in skeletal muscle, liver and adipose tissue

Both, 5 hour and 16 hour fasting of mice resulted in absence of background phosphorylation events in the insulin signalling pathway in all tissues examined (Figure 2A-F). Insulin injection for 10 minutes led to a similarly robust stimulation of insulin receptor Tyr^{1162/1163} and Tyr¹¹⁵⁸ phosphorylation sites in skeletal muscle (Figure 2A, B, *top panel*), liver (Figure 2C, D, *top panel*) and adipose tissue (Figure 2E, F, *top panel*) of both 5 hour and 16 hour fasted mice, consistent with the rise in circulating insulin levels and fall in blood glucose (Figure 1). Re-feeding did not lead to a significant increase in insulin receptor phosphorylation in either group of mice, consistent with the more modest increase in insulin levels and unchanged blood glucose levels. Similar observations were made in regards to phosphorylation of Akt/PKB and ERK1/2 in the muscle (Figure 2A, B) and liver (Figure 2C, D). However, in adipose tissue, re-feeding the mice for 45 minutes resulted in phosphorylation of Akt/PKB (Figure 2E, F), whilst Erk1/2 was not regulated by re-feeding in these mice. Both, insulin and re-feeding for 45 minutes caused a robust phosphorylation of S6 in muscle, liver and adipose tissue, in 5 hour as well as 16 hour fasted mice.

***In vivo* time course of phosphorylation events in response to insulin injection in skeletal muscle**

Since the percentage fall in blood glucose levels with insulin injection was similar in both 5 hour and 16 hour fasted mice, we performed a time course of insulin stimulation in mice with a 5 hour fast to compare to the numerous previously published studies using a 16 hour overnight fast [204 Bayascas, J.R. 2008;]. In a time-course of insulin-

stimulated phosphorylation, we found that insulin receptor phosphorylation at both Tyr^{1162/1163} and Tyr¹¹⁵⁸ sites reached a peak at 10 minutes post injection in skeletal muscle. The phosphorylation of these sites decreased slightly after 15 minutes and had returned to basal levels at 30 minute post-insulin injection (Figure 3A, B, F).

Phosphorylation of Akt/PKB (Figure 3C, F) and ERK1/2 (Figure 3D, F) reached a plateau at 15 minutes that was sustained to 30 minutes. Phosphorylation of S6 increased dramatically over the time course and did not appear to plateau or return to basal levels at 30 minutes (Figure 3E, F).

***In vivo* time course of phosphorylation events in response to insulin injection in liver**

The *in vivo* kinetics of insulin-stimulated phosphorylation events in liver was quite different to that observed in skeletal muscle, with peak insulin receptor phosphorylation at Tyr^{1162/1163} site observed at 5 minutes (Figure 4A, F) and Tyr¹¹⁵⁸ site (Figure 4B, F) at 15 minutes post-injection; as observed in skeletal muscle, IR phosphorylation on these sites was completely absent at 30 minute time-point (Figure 4A, B, F). Phosphorylation of Akt/PKB (Figure 4C, F) and ERK1/2 (Figure 4D, F) peaked at 5 minutes post-injection and remained sustained for the period of 30 minutes as observed in skeletal muscle. Phosphorylation of S6 increased dramatically over the time course and did not appear to plateau or return to basal levels at 30 minutes (Figure 4E, F).

***In vivo* time course of phosphorylation events in response to insulin injection in adipose tissue**

The *in vivo* kinetics of insulin-stimulated phosphorylation events in adipose tissue was different to that observed in skeletal muscle and liver, with peak insulin receptor phosphorylation at Tyr^{1162/1163} site observed at 15 minutes (Figure 5A, F) and Tyr¹¹⁵⁸ site at 5 minutes post-injection (Figure 5B, F); as in skeletal muscle and liver, IR phosphorylation on both sites was completely absent at 30 minute time-point (Figure 5A, B, F). Phosphorylation of Akt/PKB (Figure 5C, F) and Erk1/2 (Figure 5D, F) peaked at 5 minutes and was sustained until 15 minutes post-injection, returning to basal levels after 30 minutes of insulin stimulation. Phosphorylation of S6 increased dramatically over the time course and did not appear to plateau or return to basal levels at 30 minutes (Figure 5E, F).

Discussion

Comprehensive studies into the optimal fasting regimens to conduct glucose tolerance experiments {{200 Andrikopoulos,S. 2008; }} or euglycemic hyperinsulinaemic clamp studies {{201 Ayala,J.E. 2006; }} have been conducted. Studies examining effects of fasting and re-feeding on gene expression have also been done in the past, such as for example, studies analyzing alterations in mRNA expression levels of lipogenic genes in adipose tissue or liver with different times of fasting (0, 4, 8 and 24 hours) {{174 Palou,M. 2010; 175 Palou,M. 2008; }}. However, even though so much work is conducted analyzing the *in vivo* effects of insulin in insulin sensitive or insulin resistant states in mouse models, there have been no comprehensive studies as to the optimal fasting times or indeed the time course of insulin stimulation *in vivo* in peripheral insulin sensitive tissues. Currently, studies examining phosphorylation events in the insulin signaling pathway use different fasting protocols, which normally depend on other

published data, convenience (for example, it is easier to fast mice overnight and start a signaling experiment first thing in the morning) or susceptibility of animals to different fasting regimens. There are, however, numerous examples of mice that do not tolerate prolonged overnight fasting, as mice become severely hyperglycaemic {{169 Loh,K. 2009; }}; in addition, even in normal, healthy animals, overnight fasting leads to a state of starvation and almost complete depletion of muscle and hepatic glycogen stores {{170 Irimia,J.M. 2010; }} and utilization of fat stores. Our aim was to find out if a short term food removal from mice for just 5 hours would have the same effect as a prolonged, overnight fast in respect to circulating glucose and insulin levels, as well as basal-, insulin- and re-feeding (giving rise to physiological levels of insulin stimulation) stimulated phosphorylation events in insulin signaling pathway. We also wanted to understand if these responses were different depending on the tissue(s) being examined.

As expected, circulating glucose levels were higher in 5 hour fasted in comparison to 16 hours fasted mice. Interestingly, however, insulin injection for 10 minutes as well as re-feeding of animals for a period of 45 minutes resulted in comparable circulating glucose and insulin levels in both groups of mice. Both 5 hour food withdrawal and 16 hour fast resulted in comparable starting basal phosphorylation levels of IR, Akt/PKB, ERK1/2 and S6 in all tissues examined, suggesting that a 5 hour food withdrawal could be used for all signaling experiments. Insulin was able to stimulate insulin signaling events in both treatment groups to the same extent. Re-feeding the mice for a period of 45 minutes, thus giving rise to physiological rather than super high glucose stimulation, led to phosphorylation of S6 in both treatment groups in all tissues examined. Interestingly however, re-feeding caused phosphorylation of PKB only in the adipose tissue in both treatment groups, while skeletal muscle and liver PKB phosphorylation were unaffected, thus suggesting differential regulation of phosphorylation events in adipose tissue in comparison to the muscle and liver.

Determining the time-course of insulin-stimulated phosphorylation events in different tissues is of utmost importance, as most studies use only one time point to examine phosphorylation events in all peripheral insulin-sensitive tissues. However, certain signaling events may be missed entirely if this time course is not appropriate for the tissue(s)/target(s) being examined. It is necessary to know the time course of these events in normal healthy animals, in order to study what signaling changes may occur in an insulin resistant state. We therefore used a 5 hour food withdrawal regimen, before injecting intraperitoneally (*ip*) the normal C57Bl/6 mice with insulin for a period of up to 30 minutes, in order to examine this time course in skeletal muscle, liver and adipose tissue. Our data suggest that if examining signaling events in skeletal muscle and liver, the commonly used 10 minute *ip* injection of insulin may be appropriate especially for those interested in examining the phosphorylation status of the sites on the IR. Apart for the IR, phosphorylation of other insulin signaling components peaked at 15 minutes and was sustained for the period of 30 minutes. However, if examining phosphorylation events in the adipose tissue, most of the phosphorylation events were fast and peaked by 5 minutes post-injection, with phosphorylation events sustained up to 15 minutes and returning back to basal by 30 minutes post-injection.

It was also interesting to note that depending on the tissue(s) examined, different phosphorylation sites on the IR exhibited different time-course of phosphorylation events, such that IR Tyr^{1162/1163} peaked at 5 minutes in liver, 10 minutes in skeletal

muscle and 15 minutes in adipose tissue, while IR Tyr¹¹⁵⁸ peaked at 5 minutes in adipose tissue, 10 minutes in skeletal muscle and 15 minutes in liver. To our knowledge, this is the first report of such difference in the time-course and tissue-specificity in phosphorylation events.

Therefore, our studies have determined the *in vivo* effects of different fasting regimens on basal as well as insulin- and re-feeding-stimulated phosphorylation events and suggest that for most physiological studies, the short 5 hour food withdrawal should be used. In addition, our study is the first to investigate in depth *the in vivo* time-course of insulin-stimulated phosphorylation events in a mouse model most commonly used for insulin resistance, type 2 diabetes and obesity studies, the C57Bl/6 mouse strain. Our data suggest that careful thought must be applied to make sure that the correct time-course of insulin-stimulation should be used, which will depend on the tissue(s) being examined and signaling molecules of interest. In some cases it may mean that separate cohorts of mice may need to be generated in order to examine signaling in adipose tissue versus signaling in the liver and skeletal muscle.

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References

Methods

Animal Studies

All animal studies were performed under a project licence approved by the Home Office under the Animals (Scientific Procedures) Act 1986. Mice were maintained on a 12-hour light/dark cycle in a temperature-controlled barrier facility, with free access to water and food. To examine the effects of fasting and re-feeding regimens, thirty 10 week old male mice (C57BL/6) were fasted either for 5 hours or 16 hours (overnight), and then 5 mice from each group were injected intraperitoneally with saline or insulin (Eli Lilly; 10 mU/g body weight) or re-fed for 45 minutes. For insulin time-course experiments, thirty 10 week old male mice (C57BL/6, Charles River) were fasted for 5 hours, followed by an intraperitoneal insulin (10 mU/kg) injection for: 0, 2, 5, 10, 15 and 30 minutes. Mice in the “0” group were saline injected and sacrificed immediately. Afterwards, the mice were sacrificed by CO₂, and mouse tissues were dissected and immediately frozen in liquid N₂ for later analysis.

Metabolic measurements

Tail blood glucose was determined using a glucometer (Lifescan One-Touch Basic, Milpitas, CA). Serum insulin levels were determined by ELISA (CrystalChem) as previously described {{1 Delibegovic,M. 2007; 105 Delibegovic,M. 2009; }}.

Biochemical analysis

Whole cell lysates were prepared by extraction in RIPA buffer (10mM Tris-HCl, pH 7.4; 150mM NaCl; 0.1% SDS; 1% Triton-X100; 1% sodium deoxycholate; 5mM EDTA; 1mM NaF; 1mM sodium orthovanadate; protease inhibitors) at 4°C, followed by clarification at 14,000 x g, as described previously {{105 Delibegovic,M. 2009; 1 Delibegovic,M. 2007; 2 Bence,K.K. 2006; }}. For western blots, proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were performed with polyclonal antibodies against pIR1162/1163 or pIR1158 (Invitrogen), IR β subunit (Santa Cruz Biotechnology), pIRS1 Y608 (Calbiochem), pAKT, pGSK3, pERK1/ERK2, pS6, Akt, ERK1/2, GSK3, IRS1 (Cell Signaling) following the manufacturer's directions. Proteins were visualized using enhanced chemiluminescence (ECL), and quantified by scanning densitometry (Image J software).

Statistical analysis

Results are expressed as mean $\pm$ s.e.m. Comparisons between groups were made by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, with P values of $p < 0.05$ considered statistically significant.