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In vivo differential effects of fasting, re-feeding, insulin and insulin stimulation time course on insulin signaling pathway components in peripheral 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 signaling, 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 h fast in mice is sufficient to assess physiological responses to insulin and/or glucose in glucose tolerance tests [1], insulin tolerance tests [2–4] and euglycemic hyperinsulinemic clamp studies [5,6]. Moreover, mice are nocturnal feeders, with ~70% of their daily caloric intake occurring during the dark cycle [5], and their metabolic rate is much higher than humans. Therefore, an overnight fast in mice is closer to starvation than just food withdrawal. Thus our aim was to assess insulin signaling components from the insulin receptor to downstream targets IRS1, Akt/PKB, GSK3, Erk1/2 and ribosomal protein S6 in muscle, liver and adipose tissue in 5 h versus 16 h (overnight) fasted mice, and the time course (0–30 min) of these phosphorylation events. We also assessed whether re-feeding under 5 h and 16 h fasting conditions was a more robust stimulus than insulin alone. *Conclusions*: Our study determines that a short food withdrawal from mice, for a period of 5 h, 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.

1. 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 [7]. Autophosphorylation of these three sites is required for its maximal activation [8]. 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 [9]. 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 [10]. 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 [11]).

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 C57Bl/6J mouse model with an overnight, 16 h fast,

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before a terminal insulin signaling procedure [2–4,12,13]; however, this prolonged period of fasting induces many physiological changes, leads to a dramatic weight loss and depletes hepatic glycogen stores [14]. 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−/−) [15] whereby a 4 h fast had to be performed for the insulin signaling experiments; for other reasons perhaps, some studies also use a shorter 6 h fast, for example, experiments conducted on adipose triglyceride lipase (ATGL) deficient mice [16]. In addition, most commonly, only one time-point of insulin stimulation is used *in vivo*; with the most common one being a 10-min intraperitoneal injection, to investigate signaling events in all the peripheral 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 h) food withdrawal or a prolonged (16 h) fast,
(2) insulin injection or re-feeding,
(3) a time course up to 30 min following insulin injection.

We present here that food withdrawal from mice for a short period of time of 5 h results in a similar fasting, insulin and re-feeding response in phosphorylation events of the insulin receptor (IR), IRS1, Akt/PKB, Erk1/2 and S6 as in mice fasted for a prolonged period of 16 h in all tissues examined. In skeletal muscle only, 16 h fasting is necessary to eliminate basal phosphorylation of GSK 3. Interestingly, re-feeding the mice for 45 min results in adipose tissue phosphorylation of Akt/PKB, while it has no effect on its phosphorylation in skeletal muscle or liver. 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 min. These revealed differences in time course of phosphorylation events, with muscle and liver insulin signaling components peaking at 10–15 min post-insulin injection and remaining sustained post-IR phosphorylation, whilst adipose tissue insulin signaling peaked and returned towards basal levels after 15 and 30 min, with different time courses observed for individual signaling molecules.

2. Methods

2.1. 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-h light/dark cycle in a temperature-controlled barrier facility, with free access to water and food. To examine the effects of fasting, insulin and re-feeding regimens, thirty 10 week old male mice (C57BL/6J from Charles River) were fasted either for 5 h or 16 h (overnight), and then five mice from each group were injected intraperitoneally with saline or insulin (Eli Lilly; 10 mU/g body weight) or re-fed for a period of 45 min. For insulin time-course experiments, thirty 10 week old male mice (C57BL/6 J from Charles River) were fasted for 5 h, followed by an intraperitoneal insulin (10 mU/kg) injection for: 0, 2, 5, 10, 15 and 30 min. Mice in the "0" group were injected with saline. Afterwards, the mice were sacrificed by CO_2, and mouse tissues were dissected and immediately frozen in liquid N_2 for later analysis.

2.2. 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 [3,4].

2.3. Biochemical analysis

Whole cell lysates were prepared by extraction in RIPA buffer (10 mM Tris–HCl, pH 7.4; 150 mM NaCl; 0.1% SDS; 1% Triton-X100; 1% sodium deoxycholate; 5 mM EDTA; 1 mM NaF; 1 mM sodium orthovanadate; protease inhibitors) at 4 °C, followed by clarification at 14,000*g*, as described previously [2–4]. For Western blots, proteins were resolved by SDS–PAGE using pre-cast BioRad 18-well 10% gels and transferred to nitrocellulose membranes. Immunoblots were performed with polyclonal antibodies against pIR1162/1163 or pIR1158 (Invitrogen), IR β subunit (Santa Cruz Biotechnology), pIRS1 (Calbiochem), pAKT (Cell Signaling), pGSK3 (Cell Signaling), pERK1/ERK2 (Cell Signaling), pS6 (Cell Signaling), Akt (Cell Signaling), following the manufacturer's directions. Proteins were visualized using enhanced chemiluminescence (ECL), and quantified by scanning densitometry (Image J software).

2.4. Statistical analysis

Results are expressed as mean ± 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.

3. Results

3.1. 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 h fasting [5]. In our studies, basal insulin levels were the same in mice fasted for either 5 h or 16 h (Fig. 1A); however, basal glucose levels were 2-fold higher in 5 h fasted mice compared to 16 h fasted mice (Fig. 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 h fasted and 16 h fasted mice compared to their respective saline treated controls (Fig. 1A and B). In contrast, re-feeding for a period of 45 min, led to a more modest, physiological increase in insulin levels (~4-fold) in both, 5 h fasted and 16 h fasted mice, compared to their respective saline treated controls (Fig. 1A). In addition, blood glucose levels rose 2-fold in 16 h fasted mice but did not change significantly in 5 h fasted mice (Fig. 1B).

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

Both, 5 h and 16 h fasting of mice resulted in absence of background phosphorylation events in the insulin signaling pathway in all tissues examined apart for GSK3 which was basally phosphorylated under 5 h fasting conditions (Fig. 1C-H). Insulin injection for 10 min led to a similarly robust stimulation of insulin receptor $Tyr^{1162/1163}$ and Tyr^{1158} phosphorylation sites in skeletal muscle (Fig. 1C and D, top panel), liver (Fig. 1E and F, top panel) and adipose tissue (Fig. 1G and H, top panel) of both 5 h and 16 h fasted mice, consistent with the rise in circulating insulin

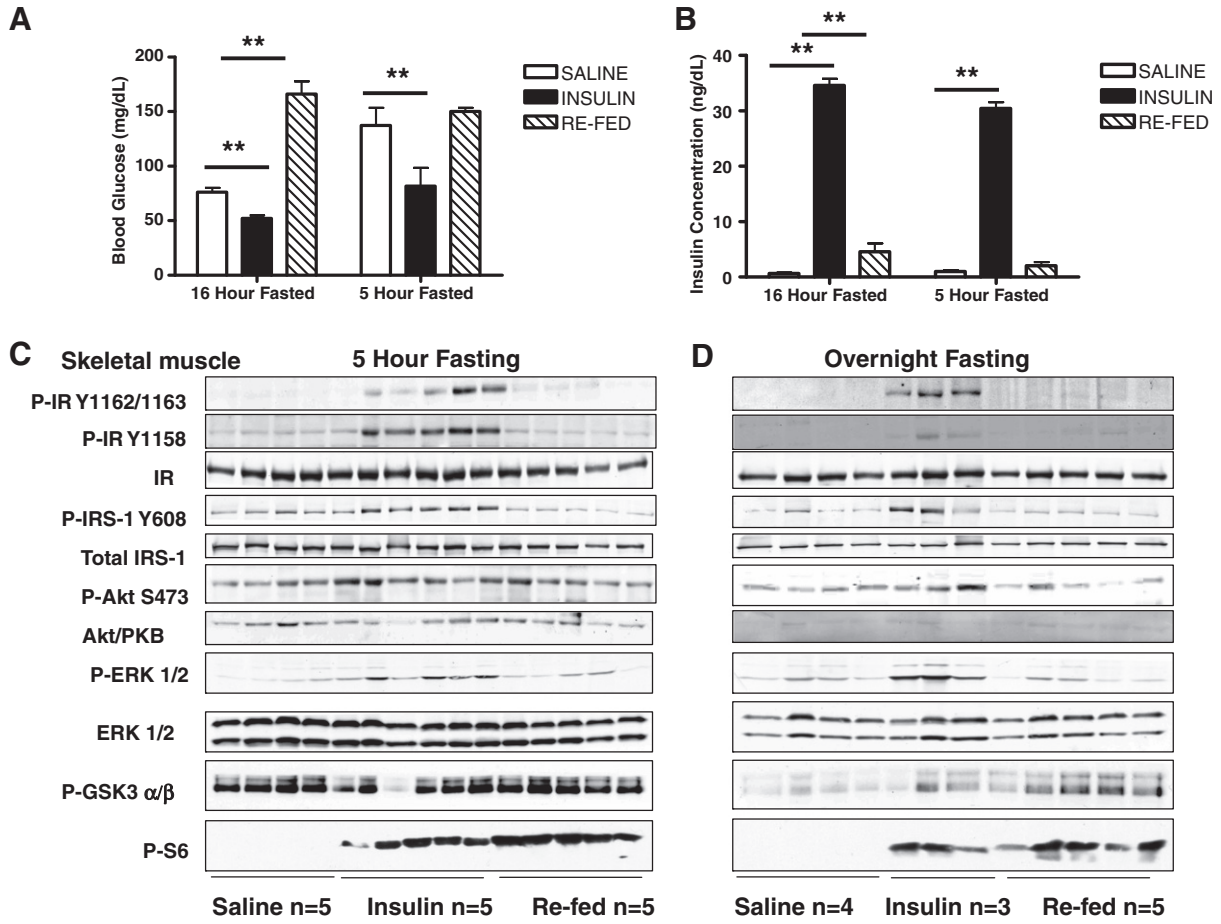

Fig. 1. (A) Insulin (ng/dL) and (B) glucose (mg/dL) levels in 5 h and 16 h fasted C57Bl/6J mice treated with saline (open bars), insulin (black bars) or re-fed for 45 min (striped bars) ($n = 5$ mice for each group); * $p < 0.05$; ** $p < 0.01$. Analysis of insulin signaling components in C57Bl/6 J mice in skeletal muscle in (C) 5 h or (D) 16 h fasted mice; liver in (E) 5 h or (F) 16 h fasted mice; or adipose tissue in (G) 5 h or (H) 16 h fasted mice.

levels and fall in blood glucose (Fig. 1A and B). 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 regard to phosphorylation of IRS1, Akt/PKB, GSK3 and ERK1/2 in the muscle (Fig. 1C and D) and liver (Fig. 1E and F). However, in adipose tissue, re-feeding the mice for 45 min resulted in phosphorylation of Akt/PKB (Fig. 1G and H), whilst Akt/PKB was not regulated by re-feeding in these mice. Re-feeding also led to a robust phosphorylation of GSK3 in muscle and liver but only after an overnight fast (Fig. 1D and H). Both, insulin and re-feeding for 45 min caused a robust phosphorylation of S6 in muscle, liver and adipose tissue, in 5 h as well as 16 h fasted mice.

3.3. 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 h and 16 h fasted mice, we performed a time course of insulin stimulation in mice with a 5 h fast to compare to the numerous previously published studies using a 16 h overnight fast [17]. In a time course of insulin-stimulated phosphorylation, we found that insulin receptor phosphorylation at both $Tyr^{1162/1163}$ and Tyr^{1158} sites reached a peak at 10 min post-injection in skeletal muscle. The phosphorylation of these sites decreased slightly after 15 min and had returned to basal levels at 30 min post-insulin injection (Fig. 2A, B and I). Phosphorylation of IRS1 at tyrosine 608 site peaked at 15 min and reached a plateu at 30 min post-injection (Fig. 2C and I). Phosphorylation of ERK1/2 (Fig. 2D, E and I) and Akt/PKB (Fig. 2F and I) reached a plateau at 15 min that was sustained to 30 min, whilst GSK phosphorylation peaked at 5 min post-injection (Fig. 2G and I). Phosphorylation of S6 increased dramatically over the time course and did not appear to plateau or return to basal levels at 30 min (Fig. 2H and I).

3.4. 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 min (Fig. 3A and F) and Tyr^{1158} site (Fig. 3B and F) at 15 min post-injection; as observed in skeletal muscle, IR phosphorylation on these sites was completely absent at 30 min time-point (Fig. 3A, B and F). Phosphorylation of IRS1 at Y608 site increased with the time course of stimulation (Fig. 3C and I). Phosphorylation of ERK1/2 (Fig. 3D, E and I), Akt/PKB (Fig. 3F and I) and GSK3 (Fig. 3G and I) peaked at 5 min post-injection and remained sustained for the period of 30 min 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 min (Fig. 3H and I).

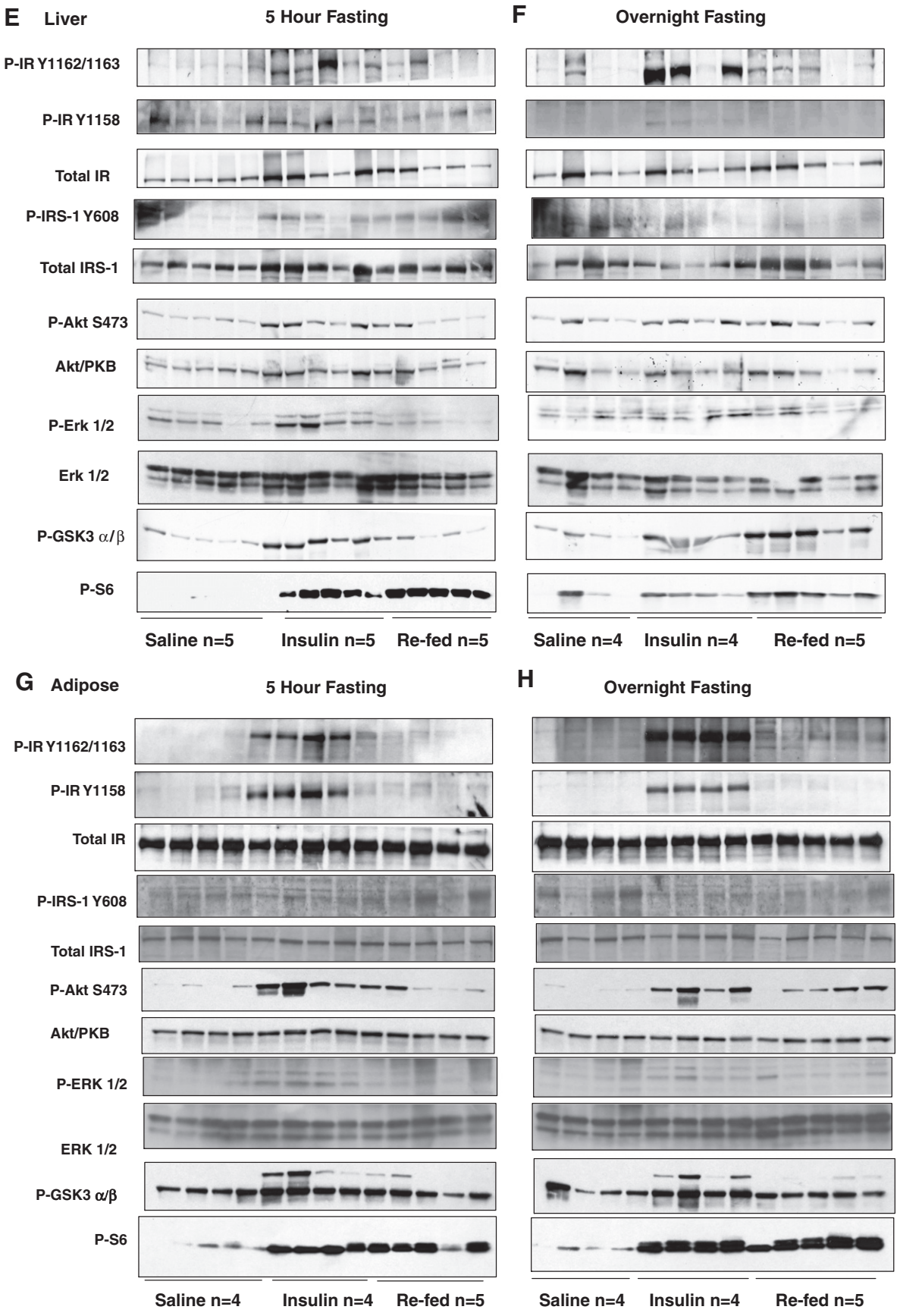

Fig. 1 (*continued*)

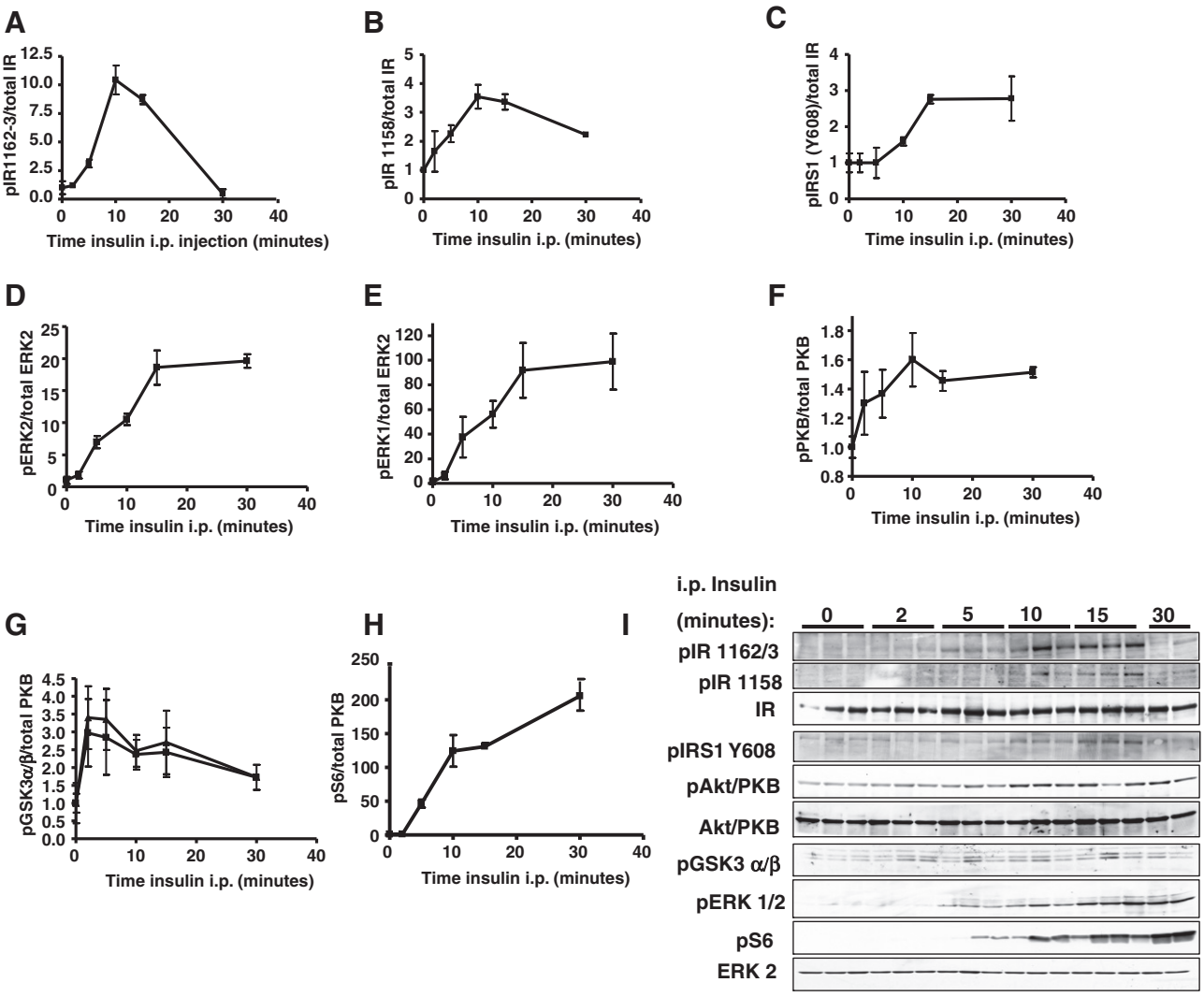

Fig. 2. Skeletal muscle insulin signaling time course for (A) phospho Insulin Receptor$^{1162/1163}$; (B) phospho Insulin Receptor^{1158}; (C) phospho IRS1 Y608 site; (D) phospho Erk2; (E) phospho Erk1; (F) phospho Akt/PKB (S473); (G) phospho GSK3; (H) phospho S6; (I) representative blots for each signaling molecule. All phosphorylated proteins were normalized against a total protein as indicated and quantified by ImageJ software.

3.5. 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 min (Fig. 4A and F) and Tyr^{1158} site at 5 min post-injection (Fig. 4B and F); as in skeletal muscle and liver, IR phosphorylation on both sites was completely absent at 30 min time-point (Fig. 4A, B and F). Phosphorylation of IRS1 at Y608 site (Fig. 4C and I), Erk1/2 (Fig. 4D, E and I), Akt/PKB (Fig. 4F and I) and GSK3 (Fig. 4G and I) peaked at 5 min and was sustained until 15 min post-injection, returning to basal levels after 30 min 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 min (Fig. 4H and I).

4. Discussion

Comprehensive studies into the optimal fasting regimens to conduct glucose tolerance experiments [1] or euglycemic hyperinsulinemic clamp studies [5] have been conducted and these found that an overnight fast of C57Bl/6J was not appropriate to detect metabolic differences but rather a shorter 5- or 6-h fasting regimen. 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 h) [18,19]. 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 peripharal 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 hypoglycemic [15]; 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 [14] and utilization of fat stores. Our aim was to

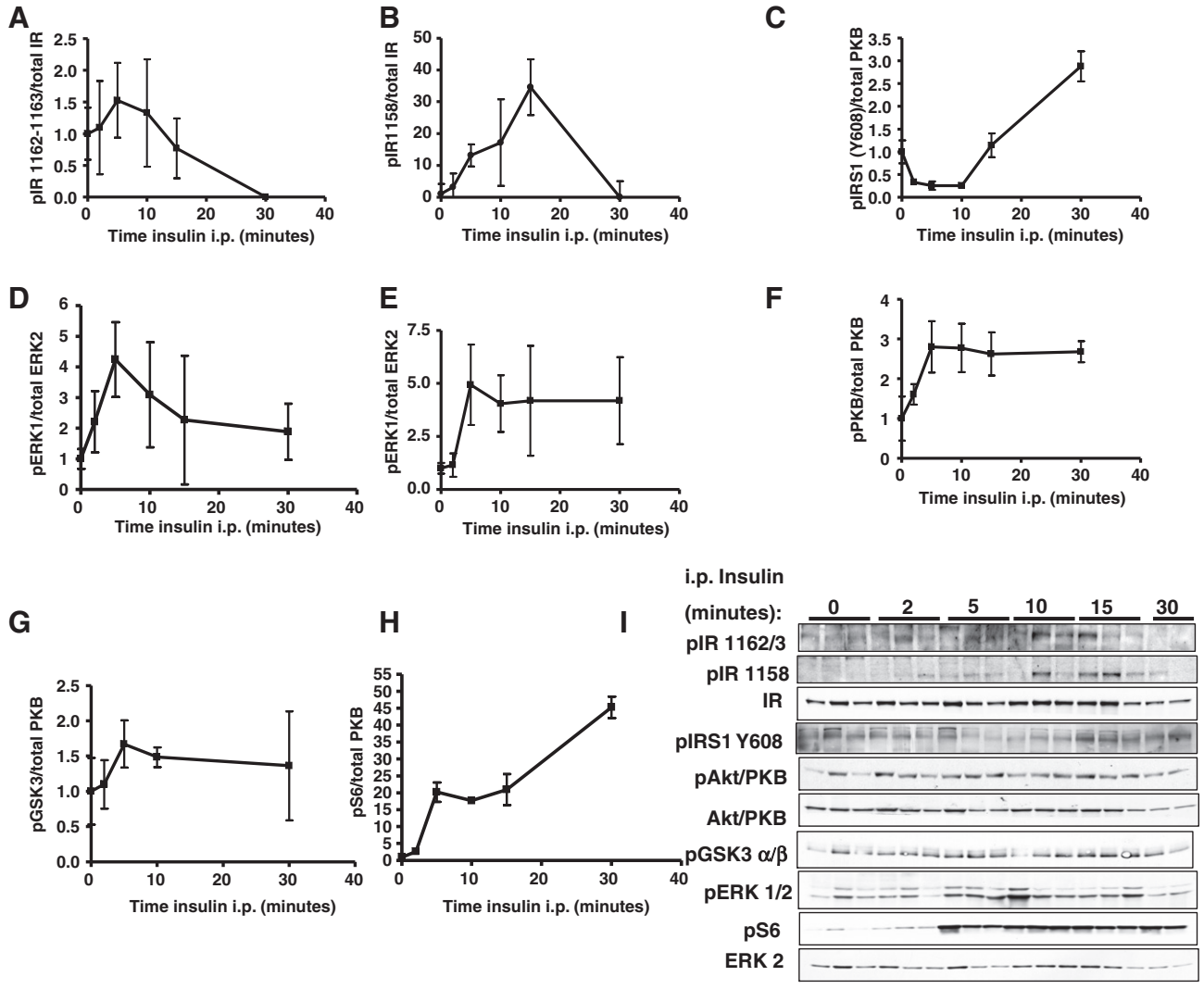

Fig. 3. Liver insulin signaling time course for (A) phospho Insulin Receptor$^{1162/1163}$; (B) phospho Insulin Receptor^{1158}; (C) phospho IRS1 Y608 site; (D) phospho Erk2; (E) phospho Erk1; (F) phospho Akt/PKB (S473); (G) phospho GSK3; (H) phospho S6; (I) representative blots for each signaling molecule. All phosphorylated proteins were normalized against a total protein as indicated and quantified by ImageJ software.

find out if a short-term food removal from mice for just 5 h 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 the 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 h fasted in comparison to 16 h fasted mice. Interestingly, however, insulin injection for 10 min as well as re-feeding of animals for a period of 45 min resulted in comparable circulating glucose and insulin levels in both groups of mice. Both 5 h food withdrawal and 16 h fast resulted in comparable starting basal phosphorylation levels of IR, IRS1, Akt/PKB, ERK1/2 and S6 in all tissues examined, suggesting that a 5 h food withdrawal could be used for all signaling experiments. The only signaling molecule that was basally phosphorylated at 5 h food withdrawal was GSK3αβ in the muscle, while there were no differences observed between two fasting regimens in liver and adipose tissue. 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 min, thus giving rise to physiological rather than super high glucose/insulin stimulation, led to phosphorylation of GSK3 in overnight-fasted muscle and liver, and S6 phosphorylation in both treatment groups in all tissues examined. Interestingly however, re-feeding caused phosphorylation of Akt/PKB only in the adipose tissue in both treatment groups, while skeletal muscle and liver Akt/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 h food withdrawal regimen, before injecting intraperitoneally (*ip*) the normal C57Bl/6 J mice with insulin for a period of up to 30 min, in order to examine the time course of insulin receptor phosphorylation and its downstream components in skeletal muscle, liver and adipose tissue. Intraperitoneal injection was used as most published literature uses *ip* injection in preference to anesthetisizing the animals and

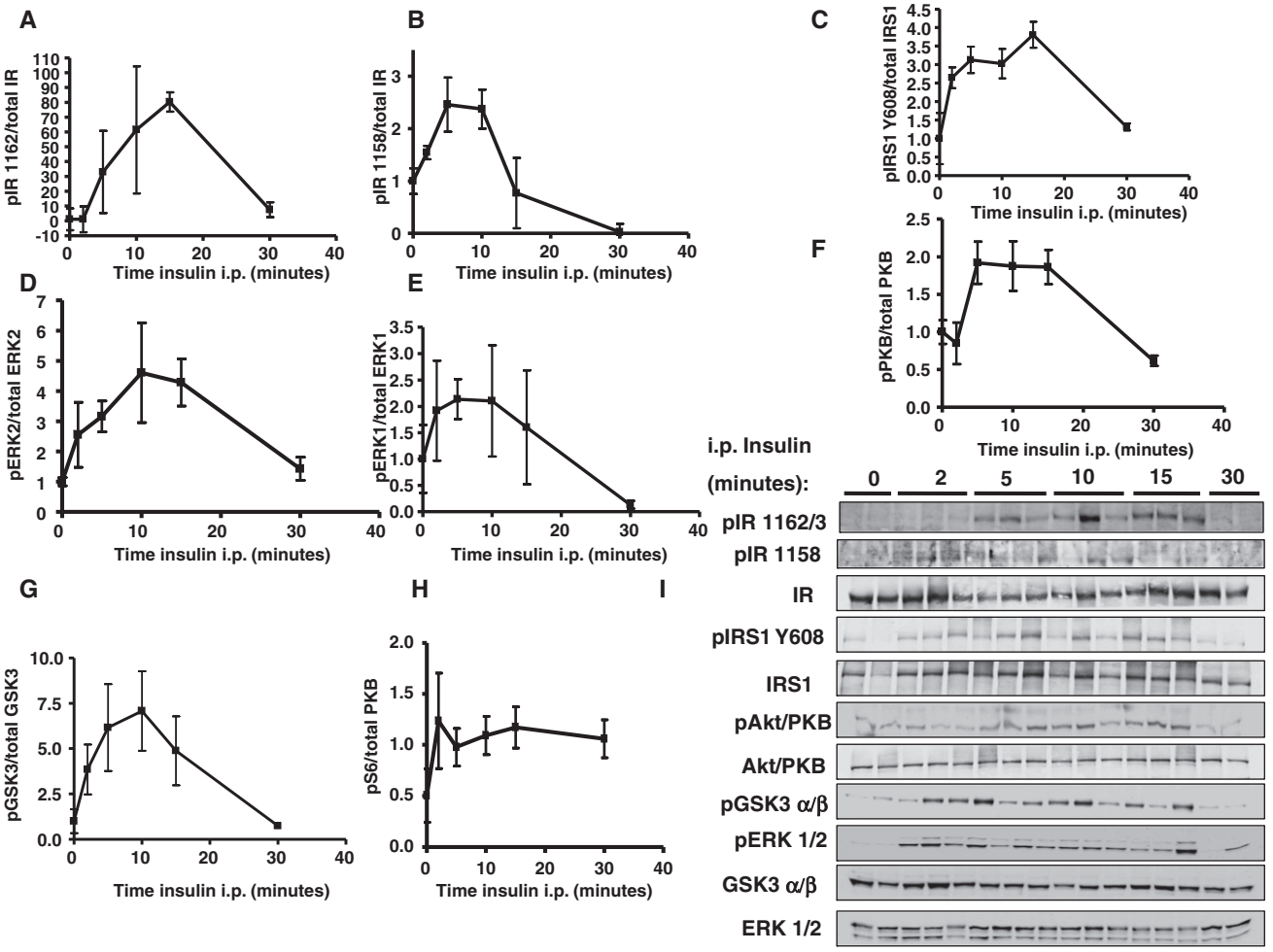

Fig. 4. Adipose tissue insulin signaling time course for (A) phospho Insulin Receptor$^{1162/1163}$; (B) phospho Insulin Receptor^{1158}; (C) phospho IRS1 Y608 site; (D) phospho Erk2; (E) phospho Erk1; (F) phospho Akt/PKB (S473); (G) phospho GSK3; (H) phospho S6; (I) representative blots for each signaling molecule. All phosphorylated proteins were normalized against a total protein as indicated and quantified by ImageJ software.

cannulating small veins. Our data suggest that if examining signaling events in skeletal muscle and liver, the commonly used 10 min *ip* injection of insulin may be appropriate especially for those interested in examining the phosphorylation status of the sites on the IR. In muscle this may still be an appropriate time course to use for other signaling molecules; however, in liver most insulin signaling components peaked at 5 min post-injection suggesting this as a more appropriate time course for liver insulin signaling analysis. In addition, if examining phosphorylation events in the adipose tissue, most of the phosphorylation events were fast and peaked by 5 min post-injection, with phosphorylation events sustained up to 15 min and returning back to basal by 30 min post-injection, again suggesting that a 5 min stimulation would be most appropriate to use.

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 min in liver, 10 min in skeletal muscle and 15 min in adipose tissue, while IR Tyr^{1158} peaked at 5 min in adipose tissue, 10 min in skeletal muscle and 15 min 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 h food withdrawal could be used, as it would avoid body mass/fat loss and depletion of glycogen stores. This however would not be appropriate if examining GSK3 phosphorylation in skeletal muscle. 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/6J mouse strain. Our data suggest that careful thought must be applied in experimental design 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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