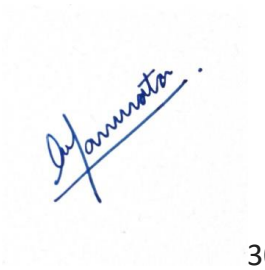
Details of the project work

Namrata Shukla: I hereby declare that the following is the original piece of work performed by me and submitted for the Sun Pharma Science Foundation Science Scholar Awards 2021



30th September 2021

Title

Insulin signalling bias beyond glycaemic control: Towards personalised interventions for diabetes

Introduction

Insulin signalling is an evolutionarily conserved signalling pathway. Right from the smallest of worms, insects and fishes to higher organisms including humans, insulin signalling is absolutely essential for regulating a multitude of biological processes from early development, growth, adult metabolism and aging. A failure in the functioning of insulin signalling causes various metabolic disorders such as diabetes, insulin resistance, cardiovascular diseases, hyper-tension and even cancer (Boucher et al., 2014; Guo et al., 2014).

Most famously known for its role in control of blood sugar, bulk insulin is released from the pancreatic cells in response to food intake. Thus, insulin is a bona fide marker of the 'fed' state i.e. food availability. This secreted insulin activates anabolic processes, which signal the organs to take up nutrients, utilise them to generate energy as well as to store for future food scarcities. These include active protein synthesis and fat storage (Saltiel and Kahn et al.,2001).

However, the function of insulin is not just limited to anabolism. It is also an active suppressor of catabolism. Catabolic processes are those which ensure that in the absence of food (like the fasted state), stored glucose and fats are broken down for energy production. Insulin

suppresses these catabolic processes to ensures no futile breakdown of stored glucose and fats happen, when food is already available in the fed state (Boucher et al., 2014; Guo et al., 2014; Saltiel and Kahn et al.,2001).

All of these anabolic and catabolic processes take place by the means of biochemical reactions, which are initiated by the binding of insulin to its receptor. Previous work in the field has been monumental in identifying more than a hundred proteins, which make this complex but co-ordinated signalling network. However, most of this research has been done using supra-physiological concentrations of insulin (~100 nM), a dose which is practically impossible in the human body (Lu et al., 2012; Haar et al., 2007).

Thus, despite a multitude of studies, we still lack the knowledge of how signalling components are integrated as a network or what parameters determine their efficient flow, under physiological concentrations of insulin (range ~0.1-1 nM) (Porksen, 2002). Additionally, given that insulin can regulate a myriad of biochemical reactions, with very different outcomes, we still don't know how one reaction is chosen over the other. Do all the reactions take place at the same time? Are some of the reactions given priority over the others? And, what regulates this complex network? Especially, with regards to insulin interventions in case of diabetes, it becomes imperative to understand how dosing and time of administration can impact this these biases in signalling. This information, in fact, could be the determining factor behind why the same insulin intervention does not work for all and pave way for personalised therapies.

This becomes even more important since insulin, despite being a 'fed' state hormone, is interestingly also secreted in the fasted state. Low dose insulin secretion (~0.1 nM) occurs in small pulses of 10-15 minutes and is essential for tissue maintenance during fasting states (Porksen, 2002). This includes protecting against both physical injury and wound healing as well as repairing cellular injury like DNA damage (Hrynyk et al., 2014; Merkel et al., 2003; Yu et al., 2017).

The importance of these fasting insulin pulses can be envisaged by the complete obliteration of this pulsatility in patients of type 2 diabetes and insulin resistance (O'Meara et al., 1993; O'Rahilly et al., 1988). However, in a healthy individual, whether this fasted pulsatile insulin

has any implications on the action of bulk insulin release in response to food availability, remains unknown.

On the other hand, repeated feeding and constant influx of high dose insulin (1 nM and <) has also been shown to cause obesity and insulin resistance.

In our current project, we employed mathematical modelling as well as experimental methods to understand how insulin concentration and frequency of administration affects the activation of diverse downstream components and brings about appropriate biological response. More specifically, we also identify mathematical parameter which elucidate selective gating of downstream component activation, depending on insulin concentration.

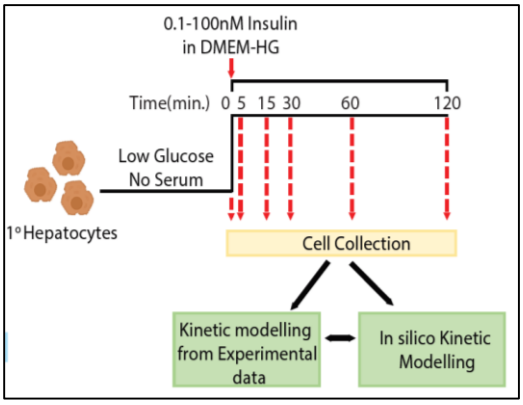
Objectives

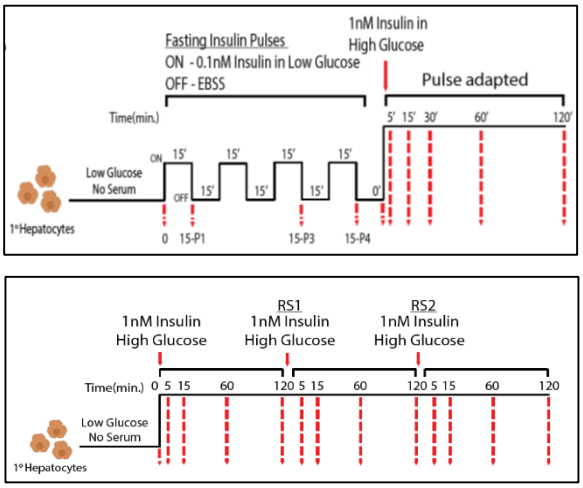
- To identify mathematical parameters which regulate insulin signalling kinetics and network under physiological concentrations of insulin
- To understand the impact of fasting state pulsatile insulin secretion on fed state insulin kinetics
- To unravel the effect of repeated insulin exposure on signalling kinetics

Material and Methods

Primary hepatocytes isolated from mice livers were used to perform all experiments. We used primary culture instead of transformed cell lines to stay as close to the *in vivo* context as possible. Hepatocytes were isolated following the protocol described in Chattopadhyay et al., 2020. Isolated hepatocytes were treated with differential concentrations of insulin (0.1-100 nM) and collected at the time points indicated (0, 5, 15, 30, 60 and 120 minutes) (Paradigm 1). For pulsatile experiments, 0.1 nM insulin pulses of 15 minutes were given before addition of fed state 1 nM insulin (Paradigm 2). For repeat insulin stimulation, 1 nM insulin was added repeatedly every 120 minutes (Paradigm 3).

Paradigm 1





Paradigm 2(top) and 3(bottom)

Cells were collected post insulin treatments and scored for activation of key components using western blotting or immunofluorescence techniques (for procedure details, see Chattopadhyay et al., 2020).

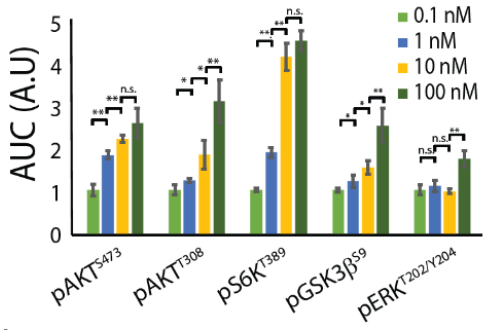
Data processing was done using Fiji-ImageJ software. Signal intensity measurements were captured and analysis was done post background correction.

Data obtained from experiments was used to mathematically model insulin signaling kinetics and further in silico modelling was performed using ordinary differential equations in Matlab (version R2018a, Mathworks). Signaling network construction, visualization and analysis was performed using Cytoscape (version 3.7.2) using Pearson correlation data obtained from GraphPad Prism (version 8).

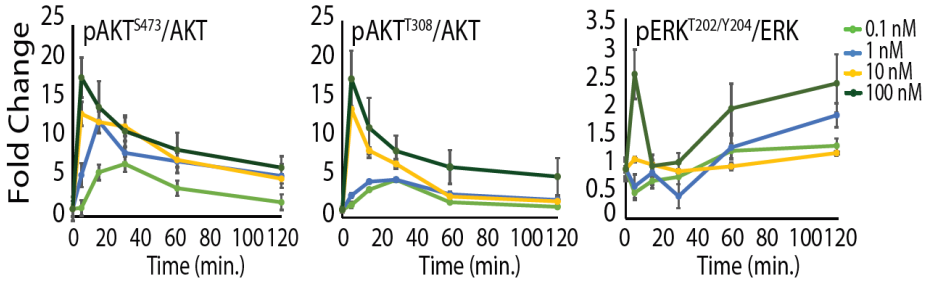
Results

1. Non-physiological concentrations of insulin generate high amplitude induction but poor signal sustenance compared to physiological doses of insulin.

In order to understand the effect of differential insulin concentration on activation of biochemical pathways, we scored for induction of key proteins downstream of insulin signalling. These included proteins involved in metabolism (AKT, GSK3β and S6K) and those involved in cellular growth (ERK). We find that while all components assessed showed a dose dependent increase in signal strength, as measured by the area under the curve (AUC), their signalling trajectories were very different.

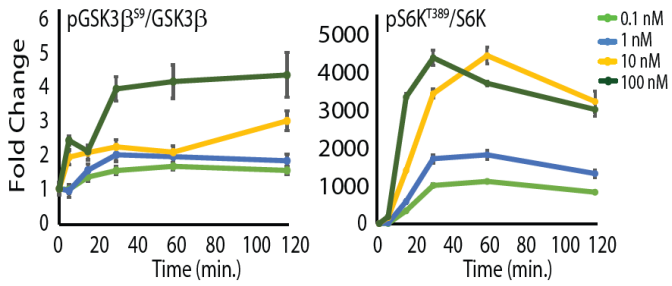


Specifically, maximal phosphorylation and its sustenance varied drastically for the nodal kinases AKT and ERK between normo- and hyper-insulinemic paradigms. While the amplitude of AKT phosphorylation decayed by 120 minutes, ERK phosphorylation showed an oscillatory behaviour with a distinct second wave of activation.



Further, physiological/normo-insulinemic concentrations (0.1 and 1nM) and supraphysiological concentrations (10 and 100 nM) displayed distinct signalling profiles. At supraphysiological concentrations, while insulin induced a rapid, high amplitude peak for AKT phosphorylations (at both T^{308} and S^{473}), there was poor signal sustenance. On the contrary, in response to normo-insulinemic treatments (0.1 and 1nM), signal sustenance was longer despite lower peak amplitude. ERK, on the other hand, displayed a flip vis-à-vis very rapid increase in phosphorylation under hyper-insulinemic states, which was not observed in response to 0.1 and 1 nM insulin treatments (wherein there was a decrease in pERK at very early time points).

Downstream of the metabolic arm, initial induction of phosphorylation at GSK3β and S6K was phase delayed in response to 0.1 nM and 1.0 nM insulin treatments and continued to remain elevated long after phosphorylation on AKT started to extinguish. While delayed response by

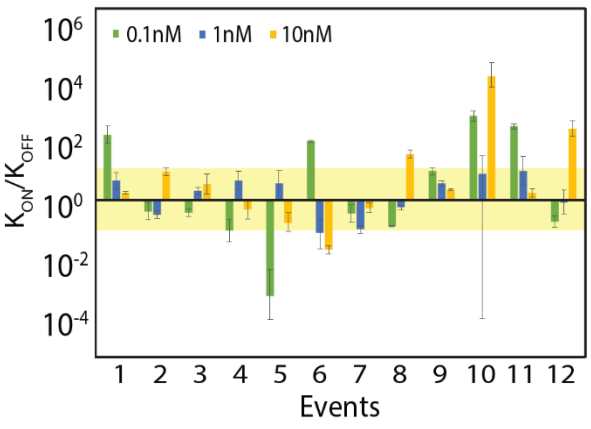


GSK3β and S6K is consistent with their downstream functions, our paradigms allowed us to delineate their signaling kinetics under both normo- and hyper-insulinemic conditions.

In totality, we observed not only differential flow of information across metabolic versus growth factor arm of insulin signalling but also difference in signalling trajectories themselves, depending on concentration of insulin.

2. Differential insulin concentrations cause selective gating of pathway components

Non-monotonic and non-linear nature of phosphorylation dynamics for different components downstream of insulin action prompted us to check how these biases in signalling were established at a systemic level. A simple way to generate bias in a biochemical reaction is to alter the rates of appearance (K_{ON}) and decay (K_{OFF}) of components. Towards this, we used our experimental data and mathematical simulations to compute the K_{ON} and K_{OFF} of individual components in the insulin signalling cascade. We assume that very high or low ratios of K_{ON}/K_{OFF} would constitute kinetic "gates" in signalling. We identify a "gate" in a



Events in the Model

Reaction No.	Chemical reaction	Event No.
1	$Ins + IR \xrightarrow{k_{f1}} pIIRC$	1
2	$pIIRC \xrightarrow{k_{b1}} Ins + IR$	
3	$pIIRC \xrightarrow{k_{d1}} \phi$	
4	$pIIRC + AKT \xrightarrow{k_{f2}} pIIRC + pAKT^{Thr308}$	2
5	$pAKT^{Thr308} \xrightarrow{k_{b2}} AKT$	
6	$pmTORC2 + AKT \xrightarrow{k_{f3}} pmTORC2 + pAKT^{Ser473}$	3
7	$pAKT^{Ser473} \xrightarrow{k_{b3}} AKT$	
8	$pIIRC + pAKT^{Ser473} \xrightarrow{k_{f4}} pIIRC + ppAKT$	4
9	$ppAKT \xrightarrow{k_{b4}} pAKT^{Ser473}$	
10	$pmTORC2 + pAKT^{Thr308} \xrightarrow{k_{f5}} pmTORC2 + ppAKT$	5
11	$ppAKT \xrightarrow{k_{b5}} pAKT^{Thr308}$	
12	$ppAKT + mTORC1 \xrightarrow{k_{f6}} ppAKT + pmTORC1$	6
13	$pmTORC1 \xrightarrow{k_{b6}} mTORC1$	
14	$pIIRC + mTORC2 \xrightarrow{k_{f7}} pIIRC + pmTORC2$	7
15	$pmTORC2 \xrightarrow{k_{b7}} mTORC2$	
16	$pmTORC1 + S6K \xrightarrow{k_{f8}} pmTORC1 + pS6K$	8
17	$pS6K \xrightarrow{k_{b8}} S6K$	
18	$pS6K + pIIRC \xrightarrow{k_{f9}} pS6K + p2IRC$	9
19	$p2IRC \xrightarrow{k_{b9}} pIIRC$	
20	$p2IRC \xrightarrow{k_{d9}} \phi$	
21	$ppAKT + GSK3b \xrightarrow{k_{f10}} ppAKT + pGSK3b$	10
22	$pGSK3b \xrightarrow{k_{b10}} GSK3b$	
23	$pIIRC + Raf \xrightarrow{k_{f11}} pIIRC + Raf^*$	11
24	$Raf^* \xrightarrow{k_{b11}} Raf$	
27	$ppERK + Raf^* \xrightarrow{k_{f12}} ppERK + Raf$	
25	$Raf^* + ERK \xrightarrow{k_{f12}} Raf^* + ppERK$	12
26	$ppERK \xrightarrow{k_{b12}} ERK$	

reaction if the K_{ON}/K_{OFF} ratio is very high or very low beyond a factor of 10 [a situation where the effective free energy/chemical potential is more than twice compared to the thermal energy ($10^{\sim e2.3}$)]. As shown below, K_{ON}/K_{OFF} ratios were determined for key phosphorylation events in insulin signaling; labelled as events 1-12 to capture phosphorylation dynamics of key components.

We find that for insulin concentration of 1 nM, which mimics a physiologically fed state, most reactions were not gated and were unlike the response to very low and very high insulin concentrations (highlighted in yellow band).

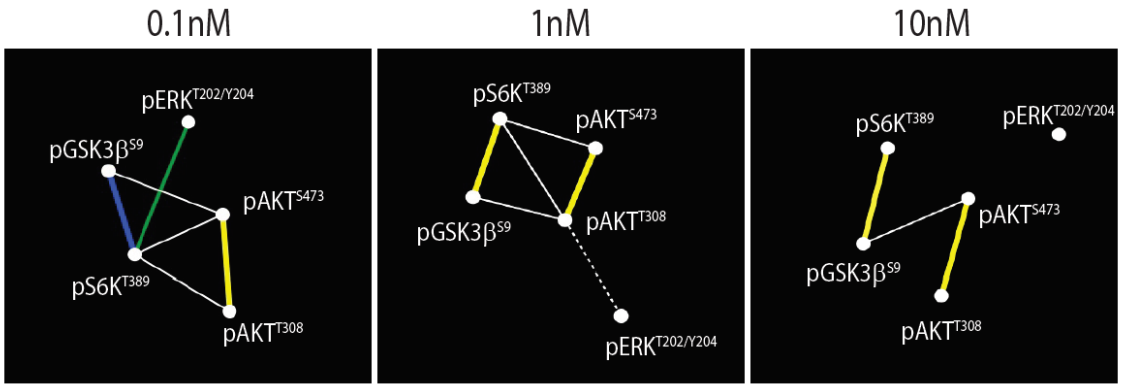
Conversely, we observed that there was a distinct uncoupling between the metabolic and growth factor arms at supraphysiological concentrations of 10 nM, with hyper-activation of cues for macromolecular synthesis (S6K activation and GSK3β inhibition, events 8 and 10, respectively), growth and proliferation (pERK formation, event 12).

Thus, physiological versus non-physiological concentrations of insulin result in differential gating of downstream signals and possibly are causal for driving biases in activation of downstream components.

3. Insulin signalling networks break at very high doses of insulin

Insulin concentration dependent differential gating of signals as well as non-concordant peak of activation and sustenance made us ask how these together dictate insulin signalling network. As can be seen from the figure below, we observed that at very high concentration of 10 nM, many components become disconnected with complete removal of ERK.

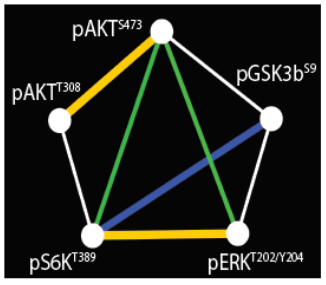
Thus, while supra-physiological concentrations of insulin generate heightened activation of downstream components, both temporally and in amplitude, the components get disproportionally correlated resulting in broken networks. Coupled with differential gating of signals, these together bring forth the need to ensure appropriate dosing of insulin, in both time and concentrations, to bring about a coordinated physiological response.

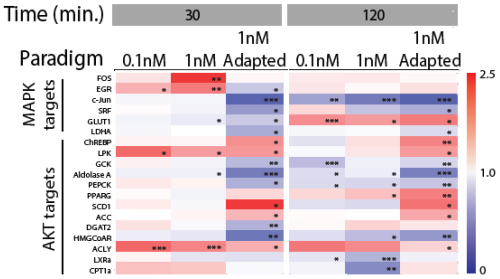


4. Low dose (0.1 nM) fasted insulin pulses sensitise response to fed (1 nM) insulin

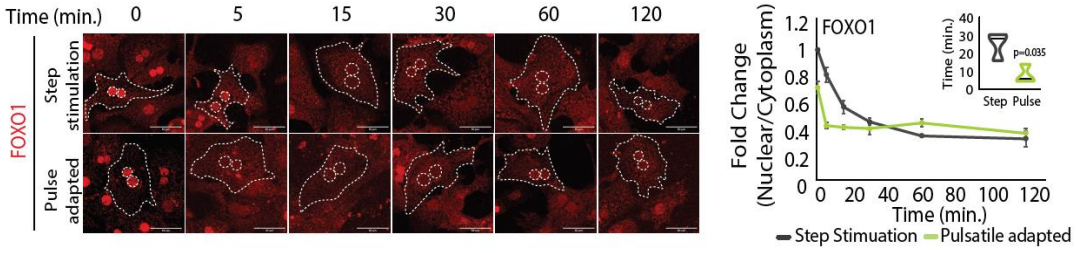
To understand the impact of pulsatility of fasting state insulin on fed state signalling, we pre-treated hepatocytes with 0.1 nM pulses of insulin before addition of 1 nM insulin. Pulse adapted hepatocytes showed more sensitised response to fed state insulin compared to non-pulsed. This was visualised by formation of more connected networks (A), robust transcriptional response, (B) as well as accelerated nuclear exclusion of AKT targeted transcription factor, FOXO1 (C). These results highlight not only the importance of insulin pulses in maintenance of cellular physiology under fasting conditions but also its crucial role in sensitising the response to fed insulin inputs.

(A) (B)





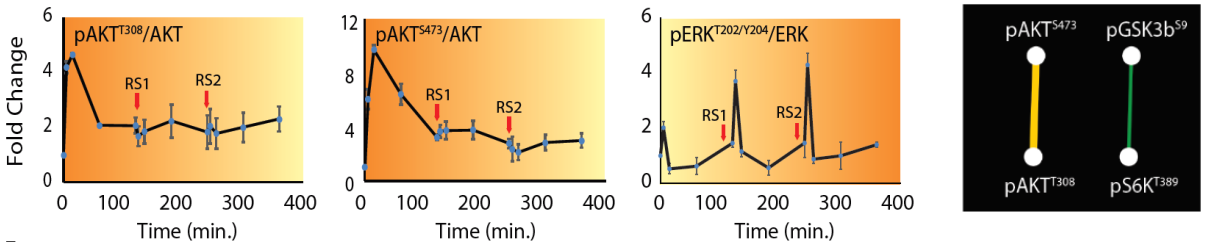
(C)



5. Repeat exposure to fed insulin (1 nM) breaks coordination between signalling components

We next wanted to assess the effect of continuous insulin exposure no signalling kinetics, a situation typically seen *in vivo*, in response to continuous feeding. Repeat exposure (RS1 and RS2) to fed state (1 nM) insulin caused disbalance between the metabolic and grow-factor arms of insulin signalling. While RS1 and RS2 failed to activate AKT (A), they caused an over-activation of ERK (B). This discordance in activation of downstream components not only disrupted transcriptional response (data not shown here) but also completely broke down the signalling network (C).

(A) (B) (C)



Statistical Analysis

All data is presented as mean ± standard error (SEM). Statistical analyses were performed using Microsoft Excel (2013). Statistical significance was determined by Student's t-test.

p-value ≤ 0.05 was considered statistically significant. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

Discussion

<u>Key highlights of the study</u>

- Physiological and supra-physiological concentrations of insulin differentially drive induction and sustenance of downstream components
- Selective kinetic gating generates bias in signalling in an insulin concentration dependent manner
- Robust signalling networks are made by physiological concentrations of insulin
- Fasting low insulin pulses sensitise signalling response to bolus insulin input in a fed state

- Repeated exposure to fed insulin doses uncouples metabolic and mitogenic arms of signalling

Novel insights into insulin signalling kinetics

Insulin signalling begins with a simple binding of insulin to its receptor and results in activation of over a hundred biochemical reactions downstream. With our study we elucidate that these biochemical reactions occur in a tightly coordinated fashion, which depends on the insulin dose and the frequency with which it is administered.

While very high doses of insulin (10-100 nM), which have been traditionally used to study insulin signalling, cause rapid and strong activation of signalling components, they result in poor co-ordination between the components. As a result, despite higher activation, the signal rapidly drops down. In a physiological setting, high doses of insulin are impossible to achieve. However, our study shows that even under some disease conditions, when insulin levels reach higher concentrations (~4-6 nM in pre-diabetes and insulin resistant states), they can potentially cause irregular activation of biochemical reactions and hence exacerbate metabolic dysregulation. Mechanistically, we identify 'kinetic gates' in the signalling network which define these differential behaviours under physiological and non-physiological concentrations of insulin.

Biomedical relevance of the study

Our study shows the importance of dose and frequency of insulin administration. We demonstrate that low dose, short pulses of insulin, which occur in typical fasted states of healthy individuals, sensitise the cell's response to insulin upon food intake. In contrast, repeated exposure to fed insulin doses (1 nM), which typically occur in response to frequent feeding or observed during diseases such as diabetes, causes resistance to carry out metabolic processes and also heightens the activity of growth factor arm. Such unequal activation of biochemical pathways has been associated with metabolic syndromes and have been postulated to be a driving force behind cancer. Our study, for the first time, shows how this occurs at the level of cellular signalling.

Over the years, insulin has undergone tremendous make-overs to make it as personalised an experience as possible. There are different kinds of insulins available, catering to the kind of

diabetes and patient requirement. However, for the longest time, when it comes to insulin dosing, only two criteria are given predominant importance: 1. It's ability to regulate blood sugar and 2. Ease of administration for the patient. Research from many years has also showed that despite so many different kinds of insulins available, many diabetics do not have desirable health benefits. In fact, many insulin dependent diabetics develop secondary complications, including obesity, tissue fibrosis, resistance to insulin itself, as well as an increased risk of cancer.

Our research indicates that such side-effects could arise from a break in co-ordination between different biochemical pathways, due to either improper dosing or frequency of administration, and strongly suggests to evaluate therapeutic insulins beyond just glycaemic control.

Impact of the research in the advancement of knowledge or benefit to mankind

The discovery of insulin in 1921 was one of the greatest leaps in the world of medical science. For the first time, we had a cure for diabetes, one of the most life-threatening diseases known.

Our study opens up new avenues to re-evaluate insulin therapy for diabetes to make it more personalised and beneficial, beyond just glycaemic control. Testing of the available insulins for their potential to differentially activate signalling components downstream of insulin action will greatly cater in choosing the right kind of insulin and frequency of administration.

Diabetes is a part of a much larger disease spectrum which includes various metabolic disorders. As most metabolic diseases overlap with both metabolic and tumorigenic pathways in the body, it is no surprise that inadequate insulin dosage and frequency of administration can result in worsening of the metabolic status and potentiate co-morbidities such as obesity, hypertension, myopathy, neuropathy etc. The study can also potentially help in treating people with a 'lean obese' phenotype, who typically look healthy but harbour a strong propensity to develop metabolic syndromes.

Together, our study not only identifies essential parameters which regulate insulin signalling at a molecular level but has huge implications in the development of truly personalised insulin interventions for ease and betterment of mankind.

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