Glucose induces an autocrine activation of the Wnt/β-catenin pathway in macrophage cell lines

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The canonical Wnt signalling pathway acts by slowing the rate of ubiquitin-mediated β-catenin degradation. This results in the accumulation and subsequent nuclear translocation of β-catenin, which induces the expression of a number of genes involved in growth, differentiation and metabolism. The mechanisms regulating the Wnt signalling pathway in the physiological context is still not fully understood. In the present study we provide evidence that changes in glucose levels within the physiological range can acutely regulate the levels of β-catenin in two macrophage cell lines (J774.2 and RAW264.7 cells). In particular we find that glucose induces these effects by promoting an autocrine activation of Wnt signalling that is mediated by the hexosamine pathway and changes in N-linked glycosylation of proteins. These studies reveal that the Wnt/β-catenin system is a glucose-responsive signalling system and as such is likely to play a role in pathways involved in sensing changes in metabolic status.

Key words: β-catenin, glucose metabolism, hexosamine pathway, N-linked glycosylation, nutrient sensing.

INTRODUCTION

One important component of the glucose homoeostatic process is the ability of key tissues to detect changes in glucose levels and to respond appropriately. There are a number of mechanisms described for how cells can sense glucose. These include ATP-sensitive potassium channels [1], AMP-activated protein kinase [2,3], activation of PKC (protein kinase C) [4] and flux through the hexosamine pathway [5]. The hexosamine pathway uses fructose-6-phosphate to generate UDP-GlcNAc (UDP-N-acetylglucosamine), which is used for both N-linked and O-linked glycosylation of proteins [4,5]. Flux through the hexosamine pathway leads to modification of various intracellular proteins with O-linked GlcNAc [6,7]. In addition, it has recently been demonstrated that flux through the hexosamine pathway can acutely regulate the dynamics of N-linked glycosylation of receptors and secreted proteins in a functional manner [8].

We have previously shown that Id2 (inhibitor of DNA binding 2), a downstream target of the Wnt/β-catenin pathway, is regulated by changes in glucose levels [9], so in the present study we sought to investigate whether the canonical Wnt signalling pathway could be regulated by glucose. Key to the transmission of canonical Wnt signals is the intracellular protein β-catenin. β-catenin is a transcriptional co-activator, and it also binds to cadherin proteins to form part of adherens junctions. The membrane-associated, cadherin-bound pool of β-catenin is highly stable. Cytosolic β-catenin is usually found in a protein complex with GSK3 (glycogen synthase kinase 3), Axin and APC (adenomatous polyposis coli). This leads to the phosphorylation of β-catenin by GSK3, which targets it for rapid ubiquitin-mediated degradation, thereby maintaining low levels of cytosolic β-catenin [10,11].

The binding of Wnts to cell-surface Frizzled receptors and LRP5/6 (low-density lipoprotein receptor-related protein 5/6) co-receptors results in a functional change in this complex such that GSK3β no longer phosphorylates β-catenin. The resultant accumulation of β-catenin in the cytoplasm leads to nuclear accumulation and binding to TCF (T-cell-specific transcription factor)/LEF (lymphoid enhancer-binding factor) transcription factors to induce the expression of specific target genes [10,11]. Many of these genes are involved in regulating cell growth and differentiation (see http://www.stanford.edu/~rnusse/wntwindow.html) and thus the Wnt signalling pathway plays an important role in development and cancer [10,12]. It is also becoming clear that the Wnt/β-catenin pathway is involved in the mechanisms regulating energy metabolism [13]. Previous studies have provided strong evidence that the Wnt/β-catenin pathway plays roles in the release of incretins from the gut [14], β-cell development [15,16] and glucose-induced insulin secretion [17]. It is also clear that Wnt/β-catenin signalling must be blocked for fat cell differentiation to occur [18–20]. Furthermore, in mice overexpressing β-catenin in liver, a number of the genes implicated in switching from gluconeogenesis to glycolysis are up-regulated [21]. Finally, compelling evidence that the Wnt/β-catenin pathway is likely to be involved in processes regulating glucose metabolism comes from genome-wide SNP (single nucleotide polymorphism) analysis studies which have identified a strong association between risk for developing Type 2 diabetes and a polymorphism in the β-catenin effector gene TCF7L2 [22,23]. Taken together these findings raise the possibility that factors that regulate signalling via the Wnt/β-catenin pathway will have an impact on metabolic pathways.

In the present study we show that physiologically relevant changes in glucose levels can regulate the canonical Wnt signalling pathway. This is mediated by the hexosamine pathway and N-linked glycosylation. This is the first evidence that...
the canonical Wnt signalling pathway is glucose-responsive, indicating that this is a previously undescribed mechanism for sensing and responding to changes in glucose concentrations.

**EXPERIMENTAL**

Unless otherwise stated, reagents were purchased from Sigma–Aldrich. The β-catenin antibody was from Symantis. Antibodies directed at phospho-β-catenin ([Ser]3/[Ser]7/[Thr]4), GS3/8, phospho-GSK3β (Ser9), phospho-LRP6 (Ser1490) and CREB (cAMP-response-element-binding protein) were from Cell Signaling Technologies. An O-GlcNAc detection kit was from Pierce. TOPFLASH and FOPFLASH constructs were kindly provided by Dr Andrew Grey (Department of Medicine, University of Auckland, New Zealand). PUGNAc [O-(2-acetamido-2-deoxy-D-glucopyranosylidene) α-naphthylcarbamate] was purchased from Toronto Research Chemicals. Recombinant mouse Dkk (dickkopf) and sFRP2 (secreted frizzled-related protein 2) were from R&D Systems.

**Cell culture and transfections**

RAW264.7 and J774.2 cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) newborn calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). HepG2 cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium; Gibco) supplemented with 10% (v/v) newborn calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). L (parental) and L-Wnt3a cells were maintained in high-glucose DMEM (Gibco) supplemented with 10% (v/v) newborn calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). Conditioned media was prepared according to A.T.C.C. guidelines. Experiments were performed on confluent cultures after overnight serum-starvation in RPMI 1640 without glucose, and supplemented with glucose as indicated. Transient transfections of RAW264.7 cells with TOPFLASH/FOPFLASH constructs were performed with FuGENETM HD transfection reagent (Roche). At 24 h after transfection, cells were serum-starved overnight in 0.5 mM glucose. A luciferase assay was performed using the Dual-Glo luciferase assay system (Promega). pRL-TK (Renilla luciferase-thymidine kinase) was co-transfected for normalization.

**Protein isolation, subcellular fractionation and immunoblotting**

Cell lysates were prepared as previously described [24]. Subcellular fractionation was performed using the NE-PER kit (Pierce). Protein concentrations were determined by BCA (bicinchoninic acid) protein assay (Pierce). Immunoblotting and visualization of immunoreactive proteins have been described previously [24].

**RNA isolation, cDNA synthesis and quantitative real-time PCR**

RNA was isolated with TRIZol® (Invitrogen). cDNA was synthesized from 2.5 μg of total RNA using Thermoscript (Invitrogen). Real-time PCR was performed using the Applied Biosystems 7900 instrument and software (Applied Biosystems). RT (reverse transcriptase) and no-template controls were included in all experiments. Primers were designed using the Primer Express Software (Applied Biosystems) and verified by melt profile analysis. SYBR green mastermix with ROX (Invitrogen) was used for PCR reactions. Gene expression was analysed by normalization to housekeeping genes ACTB (β-actin), TBP (TATA box-binding protein) and HPRT1 (hypoxanthine phosphoribosyltransferase), which were internally consistent in all samples analysed. Primer sequences used in the present study are shown below.

**Species gene primer sequence**

The primers used in the present study were: mouse Actb forward, 5′-TGGCTCAACTCTTCAGAGT-3′ and reverse, 5′-AGCTGCAATACAGTCTTCA-3′; mouse Tbp forward, 5′-ATGAGCCGATATTCTCTTGAAT-3′ and reverse, 5′-GAGTTTCTCAATGCTTCATCTCT-3′; mouse Hprt1 forward, 5′-CCCAAAATGGTTAGGTCTCAA-3′ and reverse, 5′-CTCATATTAGTCAGGCATCATC-3′; mouse β-catenin (ctnmb1) forward, 5′-TCTTCAAGCAAGCGCCTAG-3′; mouse CyclinD1 forward, 5′-GATGTGAGGGAAGAGTGTG-3′ and reverse, 5′-ACCAGGATGAAAAAGACCGTACCT-3′; mouse Axin2 forward, 5′-TCACAGGCTTTGTTGTTCAAG-3′ and reverse, 5′-GGTAGATTCCTGATGCCC-3′.

**Statistical analysis**

Results are presented as means ± S.E.M. with the number of experiments indicated in the legend. Statistical significance was assessed using one-way ANOVA and Bonferroni’s post-hoc test.

**RESULTS AND DISCUSSION**

Glucose regulates β-catenin levels in cell lines

Most cells have high levels of β-catenin due to the stable pool of β-catenin bound to cadherins which can mask any changes to the cytosolic and nuclear pools of β-catenin. Therefore, in the present study, we used the J774.2 and RAW264.7 macrophage cell lines as we have previously shown that these cells are glucose responsive [9,25] and have very low levels of classical cadherins (Figure 1A). Thus the β-catenin seen in these cell lines represents the cytosolic and nuclear pools of β-catenin that are involved in transducing Wnt signals and TCF-dependent transcriptional activation. After starvation in low glucose, restoring glucose levels led to a rapid, dose-dependent increase in β-catenin levels in both cell lines (Figures 1B and 1C). Importantly, changes in β-catenin levels were observed between 5 mM and 20 mM glucose, indicating that the effect can occur within the physiological range of glucose concentrations.

Glucose effect on β-catenin requires the hexosamine pathway

Several mechanisms have been described that allow cells to sense glucose concentrations as described above. We have previously shown that the β-catenin target gene Id2 was increased by glucose and that this effect was dependent on the hexosamine pathway [9]. In the present study we provide evidence that the hexosamine pathway is involved in regulating β-catenin levels. The evidence supporting this is: (i) the effect was blocked by azaserine and DON (6-diazo-5-oxororleucine) (Figure 2A) which are inhibitors of GFAT (glutamine:fructose-6-phosphate amidotransferase), the rate-limiting enzyme in the hexosamine biosynthetic pathway [26]; (ii) GFAT requires glutamine as an amino donor [26] and the glucose effect was lost when glutamine is not present (Figure 2B); and (iii) the effects of glucose can be replicated by low levels of GlcN (glucosamine), which can directly enter the hexosamine

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pathway downstream of GFAT (Figure 2C). Similar results were observed in J774.2 cells (results not shown).

N-linked glycosylation mediates the glucose effect on β-catenin

The hexosamine pathway produces UDP-GlcNAc, which can be used as a donor for N-linked or O-linked glycosylation of proteins [5]. To determine which of these types of glycosylation was responsible for the effect of glucose on β-catenin, we first used PUGNAc, an inhibitor of the enzyme that mediates removal of O-GlcNAc from protein. PUGNAc did not mimic the glucose effect (Figure 3A); however, in the same samples, PUGNAc did increase the overall level of O-GlcNAc (Figure 3B), demonstrating that PUGNAc is in fact inhibiting O-GlcNAc removal. We then investigated the role of N-linked glycosylation with the inhibitor tunicamycin. We found that inhibiting N-linked glycosylation with tunicamycin completely blocked the effect of glucose regulation of Wnt signalling.

Figure 1  Effect of glucose on β-catenin protein levels

(A) Western blotting was used to assess relative levels of cadherins in HepG2, RAW264.7 and J774.2 cells under growing culture conditions. β-actin served as a loading control. (B and C) Representative Western blots and densitometry quantifications of β-catenin from three independent experiments performed in triplicate expressed relative to the untreated group (means ± S.E.M.; *P < 0.05). β-actin served as a loading control. RAW264.7 cells (B) and J774.2 cells (C) were serum-starved overnight in 0.5 mM glucose and then treated for 2 h with increasing concentrations of glucose.

Figure 2 Involvement of the hexosamine pathway in the effects of glucose on β-catenin

(A) GFAT inhibitors azaserine (Aza) (10 μM) or DON (10 μM) blocked the effect of 20 mM glucose on β-catenin. (B) Glucose (20 mM) does not increase β-catenin levels in the absence of glutamine. (C) GlcN treatment mimics the effect of glucose.

(A-C) RAW264.7 cells were serum-starved in 0.5 mM glucose overnight, and treated for 2 h. Results are presented as a representative β-catenin Western blot and densitometry quantifications of three independent experiments performed in triplicate expressed relative to untreated groups (means ± S.E.M.; *P < 0.05). β-actin served as a loading control. (A) Glucose regulates Wnt signalling
Figure 3  Involvement of N-linked glycosylation in the glucose effect on β-catenin

(A–E) RAW264.7 cells were serum-starved overnight in 0.5 mM glucose and treated for 2 h as indicated. Representative Western blots and densitometry quantifications of three independent experiments performed in triplicate are shown. Values are means ± S.E.M. (*P < 0.05 compared with untreated cells). β-actin served as a loading control. (A) PUGNAc (40 μM) did not mimic the effect of low glucose, and had no effect with high glucose (20 mM). (B) PUGNAc (40 μM) does increase non-specific O-GlcNAc levels in the samples from (A). (C) Tunicamycin (10 μg/ml; Tun) blocks the glucose induction of β-catenin. (D) Increasing concentrations of 2DOG decreased β-catenin levels in low glucose and competed out the effect of 5 mM glucose (E).

Increased β-catenin protein levels with glucose treatment is the result of decreased degradation

To investigate the mechanism by which β-catenin protein levels were increased, we first examined the β-catenin transcript level. Neither glucose nor glucosamine treatments, or the absence of glutamine, affected β-catenin mRNA levels (Figure 4A). Phosphorylation of β-catenin by GSK3 is an essential step in targeting it for proteasomal degradation, so lower levels of GSK3 activity might explain increases in β-catenin [10,11]. However, we found that the total levels of GSK3β did not change with glucose or glucosamine treatment and were not affected by the absence of glutamine (Figure 4B). GSK3 is also inactivated by phosphorylation so the ratio of phosphorylated to total β-catenin is a reflection of the degree of GSK3 activity in the destruction complex. This was also unaffected by glucose or glucosamine treatment, or by the absence of glutamine (Figure 4B).

We do find, however, that the ratio of phosphorylated to total β-catenin is decreased following glucose or glucosamine treatment (Figure 4C), indicating that less GSK3β-mediated
phosphorylation of β-catenin is occurring, thus stabilizing β-catenin protein. As we found no evidence for GSK3 activity changing, the most likely explanation was that the glucose was affecting Wnt signalling as this leads to a conformational change in the destruction complex such that less GSK3-mediated phosphorylation of β-catenin occurs without any direct effect on total levels of GSK3 activity.

Glucose induces autocrine activation of the canonical Wnt signalling pathway

As there is less phosphorylation of β-catenin occurring without any effect on GSK3, we investigated whether glucose was increasing canonical Wnt signalling. Previous studies suggest mechanisms by which this could occur. Glucose could be stimulating the secretion of Wnts, as it is known that the secretion of some Wnts requires N-linked glycosylation [29,30] and autocrine regulation of Wnt signalling has been reported [31,32]. Another hypothesis is that glucose could be regulating N-linked glycosylation of the Frizzled proteins or their co-receptors (LRP5/6). This could be altering Wnt signalling as it is known that some receptors are functionally regulated by N-linked glycosylation [8]. For example, there is evidence that LRP6 is N-glycosylated and this affects the localization and signalling capacity of this receptor [33]. In each of these hypotheses, extracellular Wnt signalling would be required for the glucose effect on β-catenin. To test this we used recombinant Dkk1, which attenuates Wnt signalling by blocking the interaction between Wnts and the Frizzled–LRP complex [34]. We find that Dkk1 completely abrogates the effects of glucose on β-catenin levels (Figure 5A). The extracellular Wnt-binding protein sFRP2 also blocks Wnt signalling [35], and we find that it also blocks the effect of glucose on β-catenin (Figure 5B). Taken together these results provide evidence that the Wnt signalling pathway could be involved in the glucose effect on β-catenin.

To more directly test whether the canonical Wnt pathway was being activated by changes in glucose levels, we investigated the phosphorylation status of LRP6, as it has previously been shown that Wnt signalling at the receptor level leads to the phosphorylation of LRP6 [36,37]. As a control experiment we found that treatment of RAW264.7 cells with Wnt3a-conditioned medium induced the phosphorylation of LRP6 (Figure 5C). We went on to show that glucose induced phosphorylation of LRP6 and that this was mediated by the hexosamine pathway as the glucose effect required the presence of glutamine and could be mimicked by glucosamine (Figure 5C). Finally, glucose-induced phosphorylation of LRP6 was blocked by tunicamycin (Figure 5D), indicating that N-linked glycosylation is required for glucose-induced Wnt signalling. Taken together these results provide strong evidence that glucose-induced increases in β-catenin arise due to an autocrine activation of the Wnt signalling system, and that this involves the hexosamine pathway and N-glycosylation of proteins.

Glucose-induced changes in β-catenin leads to TCF-dependent transcriptional events

An important question was whether the increased β-catenin with glucose treatment has functional consequences for gene expression. To assess whether glucose-induced β-catenin was mediating the transcriptional events that would be expected upon Wnt stimulation, we first performed subcellular fractionation experiments and showed that glucose or glucosamine treatment led to an increase in β-catenin in the nucleus of RAW264.7 cells (Figure 6A) and J774.2 cells (results not shown). Next we demonstrated that glucose increased the transcriptional activity of a β-catenin/TCF reporter system in RAW264.7 cells (Figure 6B). Axin2 and CyclinD1 are well-recognized β-catenin target genes. We were unable to detect Axin2 mRNA in RAW264.7 cells (results not shown), but we showed that levels of CyclinD1 mRNA increased following exposure of RAW264.7 cells to glucose (Figure 6C). This glucose effect on CyclinD1 required glutamine and was mimicked by glucosamine (Figure 6C), and was blocked by tunicamycin (Figure 6D). In J774.2 cells, where both Axin2 and CyclinD1 transcripts were readily detectable, we found that glucose induced the expression of both CyclinD1 and Axin2 and this was blocked by tunicamycin (Figure 6E). Taken together these results clearly demonstrate that glucose, via the hexosamine pathway and N-linked glycosylation, increases the level of functional β-catenin, which accumulates in the nucleus and binds to members of the TCF/LEF family to activate transcription of target genes.
Implications for glucose regulation of the canonical Wnt signalling pathway

The present study provides the first evidence that physiologically relevant changes in glucose concentrations regulate β-catenin protein levels. Macrophage cell lines were used as they have low levels of cadherins and we had previously shown that Id2 levels were increased with glucose treatment in these cells. Use of cell culture models in the present study allowed dissection of the signalling mechanisms involved in the glucose effect. We find that the glucose effect is mediated via the hexosamine pathway and involves regulation of the canonical Wnt signalling system; however, we also find that β-catenin levels rapidly increase 2–3-fold in liver, muscle and fat following refeeding of fasted rats and levels are also elevated in liver of insulinopaenic, hyperglycaemic streptozotocin-induced diabetic rats (S. H. Anagnostou and P. R. Shepherd, unpublished work). This indicates both that changes in β-catenin occur in tissues involved in regulating glucose metabolism under physiologically relevant conditions and that these effects are also most likely due to changes in glucose levels in vivo.

The finding that glucose induces changes in β-catenin levels has potential implications for understanding how nutrients can regulate a range of biological processes. These mechanisms allow fine-tuning of metabolic responses to react rapidly to changes in glucose concentrations. Although a number of such mechanisms have already been described (see Introduction), several lines of evidence suggest that a mechanism involving the hexosamine pathway, regulation of Wnt signalling and changes in β-catenin levels could make an important contribution to processes regulating glucose homeostasis. First, the hexosamine pathway is known to play a role in the processes regulating glucose metabolism in muscle, liver, fat and in β-cells [5,38,39], and in the glucose-dependent expression of leptin [40]. Secondly, there is evidence that Wnt signalling plays a crucial role in regulating the differentiation of β-cells and adipocytes, both of which are key tissues involved in regulating glucose homeostasis, so any glucose-induced changes in β-catenin levels could potentially affect these processes [15,18–20,41,42]. A third line of evidence comes from the finding that, in mice overexpressing β-catenin in liver, a number of the genes implicated in switching from gluconeogenesis to glycolysis are up-regulated, in particular pyruvate kinase [21]. Changes in glucose levels are known to play a role in regulating the gluconeogenic/glycolytic switch, but the full details of the mechanisms involved are not fully understood.
We propose that changes in β-catenin levels might play a role in this process. Finally there is now strong genetic evidence that TCF7L2 plays an important role in regulating the processes involved in glucose homeostasis, and as TCF7L2 is an effector of β-catenin this also indicates that changes in β-catenin levels are likely to have an impact on glucose homeostasis [22,23].

The findings of the present study also have potential implications for understanding cancer biology. The Wnt/β-catenin pathway plays a key role in the development of many tumour types [10–12] and it is known that tumours have greatly increased levels of glucose uptake and flux through the hexosamine pathway [26,43,44]. In addition, N-glycosylation is involved in both tumorigenesis and metastasis [44–46]. This suggests that the glucose-dependent mechanism we describe could be promoting tumour growth by contributing to Wnt signalling, conceivably via the previously described autocrine Wnt signalling loop found in some cancer cells [31,32].

Together the findings of the present study provide evidence for a new regulatory mechanism by which certain cells can sense glucose levels and respond appropriately at the cellular level. Further work is required to understand whether this mechanism for regulating Wnt signalling exists in all cells, or whether it is restricted to cells involved in metabolic processes. Further analysis of this mechanism in cancer cells may lead to new approaches for targeting Wnt signalling in cancer.

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