Insulin directly stimulates VEGF-A production in the glomerular podocyte

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Insulin directly stimulates VEGF-A production in the glomerular podocyte. *Am J Physiol Renal Physiol* 305: F182–F188, 2013. First published May 22, 2013; doi:10.1152/ajprenal.00548.2012.—Podocytes are critically important for maintaining the integrity of the glomerular filtration barrier and preventing albuminuria. Recently, it has become clear that to achieve this, they need to be insulin sensitive and produce an optimal amount of VEGF-A. In other tissues, insulin has been shown to regulate VEGF-A release, but this has not been previously examined in the podocyte. Using in vitro and in vivo approaches, in the present study, we now show that insulin regulates VEGF-A in the podocyte in both mice and humans via the insulin receptor (IR). Insulin directly increased VEGF-A mRNA levels and protein production in conditionally immortalized wild-type human and murine podocytes. Furthermore, when podocytes were rendered insulin resistant in vitro (using stable short hairpin RNA knockdown of the IR) or in vivo (using transgenic podocyte-specific IR knockout mice), podocyte VEGF-A production was impaired. Importantly, in vivo, this occurs before the development of any podocyte damage due to podocyte insulin resistance. Modulation of VEGF-A by insulin in the podocyte may be another important factor in the development of glomerular disease associated with conditions in which insulin signaling to the podocyte is deranged.

podocyte; insulin; vascular endothelial growth factor-A; podocyte-specific insulin receptor knockout mice

The podocyte is the major source of VEGF-A in the glomerulus of the kidney (21). It is critical for normal kidney function both during development (10) and in maturity (9). Podocyte-derived VEGF-A is able to signal to all three cell types in the glomerulus (the glomerular endothelial cell, the mesangial cell, and back to itself) (8). Furthermore, it is now clear that there is an optimal dosage at which this molecule is beneficial for glomerular function and that both low and high levels of VEGF-A can be detrimental (10, 28). Therefore, understanding how this molecule is controlled is desirable.

Our group and others have demonstrated that the podocyte is an insulin-responsive cell in humans (5) and mice (30, 33). Furthermore, loss of podocyte insulin responsiveness in the intact perfused glomerulus results in glomerular pathology with a number of features characteristic of diabetic nephropathy (DN), including increased matrix production, glomerulosclerosis, thickening of the glomerular basement membrane, and, with time, podocyte apoptosis (33). Importantly, all of this occurs in a normoglycemic environment, demonstrating that these features are not due to glucose toxicity.

Closely related to insulin are the insulin-like growth factors (IGFs), IGF-I and IGF-II. They are structurally similar to insulin and signal predominantly through the related IGF-1 receptor (IGF-IR). It is also possible for insulin to signal through the IGF-IR and IGFs to signal via the IR, although with much less affinity for the other families’ receptor (20).

Recently, we (13) have dissected out the role of IGF signaling in the podocyte. We have shown that IGF-I and IGF-II signal predominantly through the IGF-IR and that these pathways are critically important for podocyte survival. When the receptor is stably knocked down in these cells, there is significant cell death as they mature (13).

In other cells of the body, insulin is able to directly activate cellular VEGF-A production, including in white adipose tissue (19), cardiomyocytes (3), and retinal pigment epithelial cells in the eye (18, 22). In the present study, we now show that insulin (but, interestingly, not IGFs) stimulates VEGF-A production in the podocyte in vitro and in vivo predominantly via the IR.

**METHODS**

*In Vitro Experiments*

**Cells.** Conditionally immortalized wild-type human podocytes were used as previously described (24). They were studied in their fully differentiated form after thermoswitching for 14 days, which silences the simian virus 40 transgene and allows cells to differentiate. Cells between passages 9 and 14 were studied. Recently, we have also generated conditionally immortalized podocytes from 4-wk-old mice using the same techniques as we have used in humans. These cells are responsive to insulin, with respect to phosphatidylinositol 3-kinase (PI3K) signaling as well as glucose uptake (Fig. 1). Furthermore, they express markers of mature podocytes, including nephrin, synaptopodin, and CD2-associated protein (data not shown). They adopt a differentiated phenotype 10 days after thermoswitching (the full methodology underpinning the generation and phenotyping of these cells is currently under preparation).

**Cellular stimulation.** Podocytes were starved of insulin and FCS for 12–24 h and then treated with RPMI medium alone (control) or plus insulin (10 nM) or IGF-I (100 ng/ml). Cells were stimulated with these agents for 15 min up to 24 h.

**Measurement of VEGF-A mRNA.** mRNA was prepared using TRIzol and reverse transcribed into cDNA using the reverse first-strand DNA synthesis kit (Fermentas, Burlington, ON, Canada). Expression for VEGF-A and hypoxanthine-guanine phosphoribosyltransferase (housekeeping gene) was measured in insulin-stimulated or nonstimulated cells. The primers that were used for the human and mouse experiments are shown in Table 1. The comparative ∆∆CT (where CT is threshold cycle) method of relative quantification was used to calculate for differences in gene expression using software for the ABI Prism 7900 sequence detection system (Applied Biosystems).
INSULIN, VEGF-A, AND THE PODOCYTE

Measurement of secreted VEGF-A protein. Conditioned media collected from cultured podocytes were examined using the human and murine VEGF-A ELISA DuoSet (R&D Systems) following the manufacturer’s protocol.

Short hairpin RNA knockdown of the IR and IGF-IR. Short hairpin (sh)RNA knockdown of the IR and IGF-IR was performed as previously reported (13, 33). We studied two clones with similar levels of receptor knockdown in this report: 75% knockdown for the IR and 81% knockdown for the IGF-IR.

Cell survival. Cell survival was analyzed through inspection and the use of a MTS assay to monitor cell death as previously described (13). Cell survival was analyzed through inspection and the use of a MTS assay to monitor cell death as previously described (13).

In Vivo Experiments

Podocyte-specific IR knockout mice. Podocyte-specific IR knockout mice (podIRKO) were engineered as previously described (33). For these experiments, Cre recombinase was linked to the NPHS2 (podocin) promoter. Controls were littermates who did not have the genotype NPHS2cre+/−/IRfl/fl. Mice were on a mixed genetic background. All animal work was carried out in accordance with Canadian Council on Animal Care protocols, and protocols were approved by the Animal Care Committee of Samuel Lunenfeld Research Institute.

Isolation and analysis of mouse podocytes. Isolation and analysis of mouse podocytes were achieved by studying mice at 4 wk of age, before the podIRKO mice developed albuminuria (33). Mice were crossed with transgenic neprhin cyan fluorescent protein (CFP) mice, which fluorescently tags the podocyte and allows a single cell preparation of podocytes to be isolated using FACS as previously reported (33). We pooled three podIRKO neprhin CFP+/− and compared the mRNA signal with three mice who were littermates that expressed neprhin-CFP but did not have the genotype podcre+/−/IRfl/fl. Pooling was required to isolate enough podocytes to generate adequate amounts of mRNA to be studied. mRNA analysis was performed as previously reported (33). The primers used are shown in Table 1.

\[ \text{Insulin increases VEGF-A mRNA expression within 30 min and returned to baseline by 60 min (Fig. 2A). This was translated into VEGF-A protein, which was secreted from the podocyte into the culture media. However, this was not detectable at significant levels until 24 h (Fig. 2B). Later, we also immortalized mouse podocytes from a 4-wk-old wild-type mouse using similar techniques as those used for human cells. These cells were sensitive to insulin in relation to PI3K signaling and glucose uptake (Fig. 1). They also responded to insulin with increased VEGF-A mRNA (Fig. 2C) and maximal protein production was also detectable by 24 h (Fig. 2D).} \]

We initially hypothesized that insulin could rapidly release VEGF-A protein from the podocyte, as we speculated that VEGF-A may be located in an insulin-responsive cellular compartment that would be rapidly mobilized and released in response to insulin. Therefore, we stimulated podocytes with insulin for the short timespan of 15 min and measured secreted VEGF-A protein levels in the culture media. However, we did not detect any differences in secretion at this time point when we studied either human or murine cells (Fig. 2, B and D).

Table 1. Mouse and human primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>Human VEGF-A</td>
<td>Forward 5’—CTTGACTTCCTGCTAGA-3’</td>
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<tr>
<td></td>
<td>Reverse 3’—CTAGTATTTCCCAAC-3’</td>
</tr>
<tr>
<td>Human HPRT</td>
<td>Forward 5’—GAGAATTTGTTATGC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 3’—GGCACTTGGTTTTTCT-3’</td>
</tr>
<tr>
<td>Mouse VEGF-A</td>
<td>Forward 5’—CAGGCTTCCTCTGAGA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 3’—GGGCTGGTCTGCTG3-3’</td>
</tr>
<tr>
<td>Mouse HPRT</td>
<td>Forward 5’—CCGCCTTTTTTTCG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 3’—GAATTTGCTCTCGTAC-3’</td>
</tr>
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HPRT, hypoxanthine-guanine phosphoribosyltransferase.
Previously, we (13, 33) showed that insulin predominantly signals through the IR and that IGFs signal predominately through the IGF-IR in the podocyte. Therefore, we genetically induced insulin and IGF resistance in podocytes by knocking down the major receptors through which insulin and IGFs signal, the IR and IGF-IR. In vitro, this was achieved using shRNA to produce stable knockdown cell lines of the IR (75% knockdown) and IGF-IR (81% knockdown) (33). Reduction of the IR, but not the IGF-IR, in these cells resulted in a significant decrease in VEGF-A protein production in the mature podocyte (Fig. 3A). We elected to study 81% IGF-IR knockdown cells as their magnitude of receptor knockdown was similar to the IR knockdown cells we had generated.

To extend our in vitro insulin work, we also examined the effect of podocyte insulin resistance in the intact glomerulus on VEGF-A production using an in vivo model. We studied transgenic mice in which podocytes had IRs specifically knocked down (podIRKO mice; Fig. 3B). These mice are normal at birth but develop albuminuria and progressive renal damage starting from 5 wk of age (33). Initially, we isolated
Fig. 3. Rendering the podocyte insulin resistant resulted in reduced VEGF-A production in vitro and in vivo. A: stable podocyte-specific insulin receptor (IR) knockdown (KD) cells produced significantly less VEGF-A compared with wild-type cells and also IGF-IR KD cells. n = 4–10 for each group. **P < 0.026. B: methodology used to produce single cell podocyte suspensions using FACS sorting for insulin-resistant and insulin-sensitive podocytes. CFP, cyan fluorescent protein. C: VEGF-A mRNA production in FACS-sorted insulin-sensitive and insulin-resistant podocytes ex vivo. Four-week-old mice were used. D: in situ VEGF-A production in insulin-sensitive and insulin-resistant podocytes. Representative low (×10)- and high (×40)-power images are shown, podIRKO, podocyte-specific IR knockout (KO) group. E: quantification of the intensity/glomerular area of in situ VEGF-A analysis. n ≥ 15 sections in each group. **P < 0.0011.
podocytes from these mice and examined podocyte VEGF-A production at 4 wk of age, before the development of renal disease. This revealed that VEGF-A mRNA was reduced in the podocytes of podIRKO mice compared with control mice (Fig. 3C). This was confirmed using in situ hybridization analysis of VEGF-A in glomeruli (Fig. 3, D and E). We attempted to measure glomerular VEGF-A protein expression in the mice but could not find any antibodies that reliably picked up this protein, as has also been described by other groups (31); hence, we focused on the mRNA production of VEGF-A.

Functionally, we assessed if the decreased VEGF-A production in IR knockdown podocytes affected cell survival in an autocrine fashion as VEGF-A is known to be an important survival factor for this cell type (12). However, we could not detect any differences in cell survival after 14 days of culturing of IR knockdown cells compared with wild-type cells by inspection and performing a MTS assay (data not shown). This is in contrast to the IGF-IR knockdown cells that we (13) have recently shown to exhibit significant cell death during differentiation. In IGF-IR cells, there was no detectable reduction in VEGF-A production, implying that in this situation, reduced autocrine VEGF-A action is not the major driver of cell death (Fig. 3A). We also examined the effect of IGF-I in our in vitro wild-type cell models and found that, unlike insulin, it did not elicit VEGF-A mRNA or protein production in the timeframes that insulin did (Fig. 2, A and B).

**DISCUSSION**

This study demonstrates that insulin directly increases VEGF-A mRNA and protein expression in the podocyte. Furthermore, when this cell is rendered insulin resistant, VEGF-A production is reduced. This is important as it is now clear that podocyte-derived VEGF-A is crucial to the health of the glomerulus and that its levels need to be tightly controlled as both excess (10, 32) and deficiency (9, 10) of this molecule are detrimental for glomerular function.

To our knowledge, it has not previously been shown that insulin is able to induce VEGF-A production from the podocyte. However, it has been widely reported that insulin can induce VEGF-A production in a variety of other cells throughout the body (3, 18, 19, 22, 23). It is particularly interesting that retinal pigment epithelial cells in the eye are also insulin sensitive in this respect, as they have a number of similarities to podocytes. These include that they are separated by a basement membrane from endothelial cells, to which they signal (15), and that both of these cell types are early targets in the development of major diabetic complications (nephropathy and retinopathy). In the eye, intensification of insulin early in diabetes results in increased synthesis of VEGF-A from retinal pigment epithelial cells (18, 22), which causes neovascularization. This is clinically important as local administration of anti-VEGF-A therapies can prevent this neovascularization from progressing (22, 26). Furthermore, the kinetics and magnitude of the effect of insulin’s actions on VEGF-A production in the podocyte are very similar to those demonstrated for retinal pigment epithelial cells (18). This is particularly relevant for the speed of increased VEGF-A mRNA expression in response to insulin, as we found, in both humans and mice, this to be a relatively rapid effect, within 2 h. This suggests that its mechanism of action may be different in these perivascular cells compared with other cell types, in which insulin takes longer to increase VEGF-A mRNA levels (2, 34). Going forward, it will be of interest to understand the mechanism of increased podocyte VEGF-A mRNA expression in response to insulin, as it could be due to increased transcription or decreased mRNA breakdown of VEGF-A.

In podocytes, this work demonstrates that insulin does not rapidly change the amount of VEGF-A protein secreted by the cell, as shown in our study by no detectable increase in the release of VEGF-A into the culture media after 15 min of insulin stimulation, but rather alters the “steady-state” production of this molecule. This is detectable at the protein level by 24 h for both human and mouse cell types. This is in contrast to some of the other cellular effects that insulin has on the podocyte, which have been shown to occur rapidly, within minutes. These include glucose uptake [via glucose transporter translocation (5)], actin remodeling (33), and translocation of ion channels into the plasma membrane (16, 17).

The modulation of VEGF-A release from the podocyte by insulin may be clinically important in a number of settings where cellular insulin signaling is altered and glomerular disease develops. These include chronic renal insufficiency, which is associated with cellular insulin resistance (7) and also DN, which is the leading cause of renal failure in the world. In DN, there is evidence that glomerular VEGF-A can increase (4, 14) and decrease (1, 25, 27) and that this may be related to the duration of diabetes (with an increase early on). Furthermore, it has now been shown that therapeutically manipulating VEGF-A in DN can be beneficial in some settings by both suppression (6, 11) and, conversely, supplementation (29). This suggests that there is a therapeutic window in which it has maximal beneficial effects. It is possible that the insulin responsiveness of the podocyte could at least partially govern the amount of VEGF-A release from the podocyte, and, hence, the relative insulin responsiveness of this cell could be affecting glomerular function through this mechanism. However, there must be other important modulators of podocyte VEGF-A production as there are experimental settings where early in

![Effect of high glucose (25mM) on podocyte VEGF production levels](http://ajprenal.physiology.org/)
type I diabetes there is increased podocyte VEGF-A production with associated relative insulinopenia (4). An attractive modulator is elevated glucose exposure in this setting, but we have found that rather than increasing VEGF-A release, prolonged glucose exposure suppresses VEGF-A, making this unlikely (Fig. 4).

The functional significance of reduced VEGF-A podocyte production in insulin resistance is currently unclear. We initially speculated that a chronic reduction in podocyte VEGF-A in insulin resistance could be having detrimental for this cell’s survival as it is has been shown to be an autocrine cell survival factor (12). However, our in vitro models with the IR knocked down by 75% did not find any evidence of cell death occurring. This contrasted with cells having a similar level of knockdown of the IGF-IR (81%), which showed significant cell death during cell maturation (13) but no reduction of VEGF-A production. It is therefore clear that VEGF-A is not the only important survival pathway elicited by insulin/IGF signaling.

One possibility is that in our in vitro models we did not allow enough time for cell death to become apparent and perhaps prolonged VEGF-A reduction triggers cell death. Additionally, we only knocked the IR down by 75%, and, although we (33) have shown that this abrogates insulin-stimulated PI3K and MAPK signaling pathways in these cells, we may not have rendered the podocyte sufficiently insulin resistant to observe a cell death phenotype through reduced VEGF-A secretion in our model.

Finally, it is also possible that reduced VEGF-A production by the podocyte has detrimental glomerular paracrine effects on other cell types of the glomerulus (mesangial cells and glomerular endothelial cells), as has previously been shown (8).

In conclusion, we demonstrated that insulin sensitivity of the podocyte is important in controlling VEGF-A release from this cell and that when this cell is rendered insulin resistant, VEGF-A production is suppressed. This may be relevant in the development of glomerular disease under conditions in which insulin signaling to the podocyte is deranged.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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