Fetuin-A write up

1. NF-κ B mediates lipid-induced fetuin-A expression in hepatocytes that impairs adipocyte function effecting insulin resistance. Biochem. J. (2010) 429, 451–462.
   Fetuin-A, [α_2 Heremans–Schmid glycoprotein (AHSG)] is a hepatokine that acts as an endogenous inhibitor of insulin receptor tyrosine kinase and has recently been implicated in insulin resistance (IR) and Type 2 diabetes (T2D). Fetuin-A-knockout mice exhibit improved insulin sensitivity. The human fetuin-A gene, located on chromosome 3q27, has been identified as the T2D susceptibility locus. Although fetuin-A is poised to be a potential factor affecting IR and T2D, little is known about the regulation of its synthesis in the liver, although an influence of lipids has been suggested. In subjects with NAFLD leading to T2D, a significant increase in fetuin-A levels and its mRNA expression is seen, whereas a decrease in liver fat does the opposite. We report that NEFA and fetuin-A coexist at high levels in the serum of db/db mice. Incubation of HepG2 cells and rat primary hepatocytes with palmitate enhanced fetuin-A secretion more than 4-fold over the control. The cell lysates showed overexpression and activity of NF-κB. In NF-κB-knockout HepG2 cells, palmitate failed to increase fetuin-A secretion, whereas forced expression of NF-κB released fetuin-A in the absence of palmitate. Palmitate stimulated NF-κB binding to the fetuin-A promoter resulting in increased reporter activity. These results suggest NF-κB to be the mediator of the palmitate effect. Palmitate-induced robust expression of fetuin-A indicates the presence of additional targets. We found fetuin-A to severely impair adipocyte function leading to IR. Our results reveal a new dimension of lipid-induced IR and open up a target for therapeutic intervention in T2D.

2. Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. Nature Medicine 2012 Aug;18(8):1279-85.

   Toll-like receptor 4 (TLR4) plays a key role in innate immunity by activating inflammatory signaling pathways. Free fatty acids (FFAs) stimulate adipose tissue inflammation through the TLR4 pathway, resulting in insulin resistance (IR). However, FFAs do not directly bind to TLR4 but an endogenous ligand for TLR4 remains to be identified. Here we show that fetuin-A (FetA) could be this endogenous ligand and that it has a crucial role in regulating insulin sensitivity via Tlr4 signalling. FetA knockdown in high-fat diet (HFD) fed mice with IR caused downregulation of Tlr4-mediated inflammatory signaling in adipose tissue, whereas selective administration of FetA induced inflammatory signaling and IR. FFA-induced proinflammatory cytokine expression in adipocytes occurred in the presence of both FetA and Tlr4; removing either of them prevented FFA-induced IR. We further found that FetA, through its terminal galactoside moiety, directly binds the residues of Leu100–Gly123 and Thr493–Thr516 in Tlr4. FFAs did not produce IR in adipocytes with mutated Tlr4 or galactoside-cleaved FetA. To summarize, FetA fulfills the requirement of an endogenous ligand for TLR4 through which lipids induce IR. This may position FetA as a new therapeutic target for managing IR & T2D.

   As the liver is a major source of FetA, we partially hepatectomized rats to lower the serum FetA levels as an alternative means to genetically knocking down its expression. We observed a significant decrease in serum FetA levels along with a reduction in pNf-kB, Il-6 and TNF-α in partially hepatectomized HFD rats as compared to control HFD rats. Adipose tissue from lipid-infused FetA-/- mice did not show Tlr4 activation, whereas it was apparent in wild-type (WT) mice. Glucose and insulin tolerances were decreased, and insulin resistance was increased after palmitate infusion in WT mice, whereas in FetA-/- mice these parameters were unchanged.
   These results implicate three important possibilities regarding the involvement of FetA and Tlr4 in lipid-induced insulin resistance:
   a. the decrease in Nf-kB phosphorylation in the absence of FetA may be due to impaired Tlr4 signaling
   b. insulin resistance due to lipotoxicity requires the presence of both Tlr4 and FetA
   c. a physical association between FFAs, FetA and Tlr4 is likely to be linked to insulin resistance.

To address the first possibility, we investigated whether FFA-induced Tlr4 signaling requires FetA. Palmitate had maximum binding to FetA and produced the highest inflammatory response in 3T3L1 mouse adipocytes compared to other FFAs. We therefore incubated human adipocytes or 3T3L1 cells with palmitate, FetA and both in the absence or presence of CLI-095, a TLR4 signaling inhibitor, followed by determination of NF-kB activity through western blotting, chromatin immunoprecipitation (ChIP) and reporter assays. FFA-induced activation of NF-kB and elevated expression of the cytokines IL-6 and TNF-α occurred only in the presence of FetA, whereas CLI-095 incubation or silencing of Tlr4 expression by siRNA prevented this activation and cytokine expression. These results were notable given previous reports suggesting that FFAs activate the Tlr4 signaling pathway in 3T3L1 adipocytes. One possibility for this disparity could be the high FetA concentration in FBS. As commercially available FBS contains ~20 mg ml−1 FetA, conventionally used culture media (with 10% FBS) have a substantial amount of FetA, which might allow exogenously added FFAs to induce Tlr4 activation. We therefore used serum-free medium in our experiments. FFAs alone had a marginal stimulatory effect that seemed to be independent of Tlr4 because addition of CLI-095 to the medium did not alter the response to exogenous FFAs. That FFA can induce inflammatory signaling pathways independent of Tlr4 influence has also been shown by others. In contrast, stimulation of Nf-kB activation by FetA was inhibited by CLI-095, indicating that FetA's effect is mediated through Tlr4.

We made similar observations when we incubated macrophages and adipocytes from Tlr4−/− mice with FetA and saw that FetA did not activate the Tlr4 pathway, thus indicating the requirement of Tlr4 for FetA's effect on Nf-kB activation. We also evaluated FFA plus FetA (FFA+FetA) induction of TLR4 signaling by PCR array and found that gene expression of various pro-inflammatory cytokines in human adipocytes belonging to the TLR4 pathway were greatly upregulated, whereas FFA alone produced a marginal effect. This was also reflected in the insulin sensitivity assay, wherein FFA+FetA showed a significantly greater inhibitory effect on insulin-stimulated [14C]-2DOG uptake by 3T3L1 adipocytes as compared to FFA or FetA alone.

Because MyD88) and MEK are downstream of theTLR4 signaling pathway, failure of Nf-kB activation in MyD88-knockout 3T3L1 adipocyte cells incubated with FFA+FetA or in cells preincubated with the MEK inhibitor U0126 suggest that FFA+FetA acts through the Tlr4 pathway. FFA+FetA experiments with macrophages also had similar results; FFA augmentation of Tlr4 activation was dependent on Fet-A, underpinning a link between insulin resistance and innate immunity. We examined this further in primary cell cultures of macrophage and hepatocytes obtained from standard diet–fed Tlr4-/- mice, in which FetA alone failed to induce Nf-kB activation but did so in standard diet–fed WT mice. We obtained similar results with HFD-fed insulin-resistant mice, suggesting that Tlr4 is necessary to produce the FFA+FetA effect on NF-kB activation. From these results it would seem evident that FetA is required for FFA-TLR4 signaling. However, we have observed that FFAs bind weakly to TLR4, in absence of FetA. In a lipid-protein overlay assay, FFAs bound FetA but not TLR4, suggesting that FetA acts as an intermediary between FFA and TLR4. Indeed, CHIP assay revealed the existence of a complex between FetA and TLR4.

To study the nature of this complex and to probe the possibility of a physical interaction between FetA and TLR4, we performed the surface plasmon resonance (SPR) experiment in which varied concentrations of FetA were flowed over a histidine-tagged human TLR4-MD2 complex immobilized on a nitrilotriacetic acid (NTA) sensor chip. The resulting sensorgram showed concentration-dependent high affinity binding of FetA to TLR4, KD value ($1.8 \times 10{-}8$ M). Reversing the situation, wherein FetA remained immobilized on the carboxymethylated dextran (CM5) chip and TLR4 was used as analyte, produced a similar binding affinity ($KD = 2.3 \times 10{-}8$ M). The inability of high mobility group A1 (HMGA1) to interact with TLR4 indicates the specificity of this interaction.

To examine whether such a complex between FetA and TLR4 is operative in an intracellular system, we performed a yeast two- hybrid (Y2H) assay using pGBKT7 fused with DNA encoding the extracellular domain or cytosolic domain of Tlr4. These plasmids were separately transfected into the Y2H Gold strain of yeast, followed by mating with the Y187 strain containing full-length FetA inserted into pGADT7. FetA interacted with the extracellular domain of Tlr4, as interaction-dependent growth

of colonies was observed in SD/−Ade/−His/−Leu/−Trp (quadruple dropout, QDO) plates, whereas colony growth did not occur after mating with yeast express ing the plasmid encoding the cytoplasmic domain of Tlr4. We then searched the probable sites of the TLR4 extracellular domain to which FetA binds. We presumed it could be the region of leucine-rich repeats (LRR), as those have been previously found to be involved in protein-protein interactions. To analyze FetA recognition of specific LRR sites, we mutated different LRRs in Tlr4. Deletion mutation of LRR2 (Leu100–Gly123) or LRR6 (Thr493–Thr516) in the extracellular domain of Tlr4 impeded its interaction with FFA+FetA, as indicated by diminished Nf-kB luciferase activity and cytokine expression in differentiated adipocytes and by α-galactosidase activity in Y2H studies. Deletion mutations at these sites not only restored but also augmented insulin sensitivity, possibly because of the dominant- negative nature of the mutations. Mutational studies suggest these two LRRs of Tlr4 as the crucial sites where FetA binds and permits FFAs to exert their deleterious effect on insulin activity. It is possible that FFAs, FetA and TLR4 form a complex. To examine this, we incubated [3H]-palmitate with FetA followed by human TLR4 incubation. Both Coomassie staining and flurography of the native-PAGE gel revealed a ternary complex between the three of them.

To determine the biological relevance of FetA-TLR4 interactions in FFA-induced insulin resistance, we used genetically engineered HEK- Blue hTLR4 cells, containing a TLR4–NF-kB–secreted embryonic alkaline phosphatase (SEAP) reporter system to detect TLR4–NF-kB activation. Incubation of FFAs in presence of FetA enhanced SEAP activity, inhibited by CLI-095, in a dose and time dependent, indicating that FFA+FetA operates through the TLR4 pathway. Immunofluorescence study further supported the notion that both FetA and TLR4 are required to transduce FFA signals, because FFA+FetA-stimulated nuclear translocation of NF-kB was abrogated by CLI-095. We identified the sequence of TLR4 where FetA binds; however, which part of FetA recognizes the TLR4 sequence remained unknown. As terminal carbohydrate moieties of glycoproteins such as sialic acid, galactose and fucose underpin cell adhesion, receptor recognition and host-pathogen interactions, we presumed that the glycan moieties of FetA bind TLR4, especially as Gram- negative bacteria trigger TLR4 signaling through the glycan residues of lipopolysaccharide. To investigate this possibility, we used various enzymes to cleave specific glycosidic and amide bonds in FetA. Truncated FetA and FetA's glycan part, obtained by enzymatic cleavage, were separately added to HEK-Blue hTLR4 cells. To determine the biological relevance of FetA-TLR4 interactions in FFA-induced insulin resistance, we used genetically engineered HEK- Blue hTLR4 cells, which contain a TLR4–NF-kB–secreted embryonic alkaline phosphatase (SEAP) reporter system to detect TLR4–NF-kB activation. Incubation of FFAs in the presence of FetA enhanced SEAP activity, which was inhibited by CLI-095, and this was dose and time dependent, indicating that FFA+FetA operates through the TLR4 pathway. Immunofluorescence study further supported the notion that both FetA and TLR4 are required to transduce FFA signals, because FFA+FetA-stimulated nuclear translocation of NF-kB was abrogated by CLI-095. We identified the sequence of TLR4 where FetA binds; how- ever, which part of FetA recognizes the TLR4 sequence remained unanswered. As terminal carbohydrate moieties of glycoproteins, such as sialic acid, galactose and fucose, underpin cell adhesion, receptor recognition and host-pathogen interactions, we presumed that the glycan moieties of FetA bind TLR4, especially as Gram- negative bacteria trigger TLR4 signaling through the glycan residues of lipopolysaccharide. To investigate this possibility, we used various enzymes to cleave specific glycosidic and amide bonds in FetA, as indicated. Truncated FetA and FetA's glycan part, obtained by enzymatic cleavage, were separately added to HEK-Blue hTLR4 cells. To determine the biological relevance of FetA-TLR4 interactions in FFA-induced insulin resistance, we used genetically engineered HEK- Blue hTLR4 cells, which contain a TLR4–NF-kB–secreted embryonic alkaline phosphatase (SEAP) reporter system to detect TLR4–NF-kB activation. Incubation of FFAs in the presence of FetA enhanced SEAP activity, which was inhibited by CLI-095, and this was dose and time dependent, indicating that FFA+FetA operates through the TLR4 pathway. Immunofluorescence study further supported the notion that both FetA and TLR4 are required to transduce FFA signals, because FFA+FetA-stimulated nuclear translocation of NF-□B was abrogated by CLI-095. We identified the sequence of TLR4 where FetA binds; how- ever, which part of FetA recognizes the TLR4 sequence remained unanswered. As terminal carbohydrate moieties of glycoproteins, such as sialic acid, galactose and fucose, underpin cell adhesion, receptor recognition and host-pathogen interactions, we presumed that the glycan moieties of

FetA bind TLR4, especially as Gram- negative bacteria trigger TLR4 signaling through the glycan residues of lipopolysaccharide. To investigate this possibility, we used various enzymes to cleave specific glycosidic and amide bonds in FetA, as indicated. Truncated FetA and FetA's glycan part, obtained by enzymatic cleavage, were separately added to HEK-Blue hTLR4 cells. □-galactosidase– or PNGaseF-cleaved FetA was unable to stimulate SEAP activity. Given that these results coincided with lower Nf-kB promoter activity and a concomitant reduction of proinflammatory cytokines expression, the terminal alpha-galactoside moiety of FetA seems crucial for recognizing TLR4. We show that alpha-galactosidase–cleaved FetA did not produce FFA-induced insulin resistance in adipocytes.

The influence of dietary lipids on the activation of the TLR4–NF-kB pathway to produce proinflammatory cytokines, resulting in insulin resistance, has long been known. The amount of fat in HFD could be a factor in TLR4-mediated insulin resistance; 45% fat in HFD has been shown to be insufficient to produce such response, whereas 55–60% fat in HFD effected insulin resistance through TLR4. We used HFD with 65% fat, which clearly induced insulin resistance. However, the molecular pathways that underlie an FFA-TLR4 interaction continue to elude investigators. FetA is a major carrier protein of FFAs in the circulation, and in the absence of evidence showing direct binding of FFAs to TLR-4, we envisioned an endogenous presenter of FFAs to TLR4. We have shown here that FetA serves this purpose, as it physically interacts with both FFAs and TLR4. Moreover, our work indicates a key role of FetA in FFA-induced TLR4 activation in adipocytes and thus in insulin resistance. These findings further suggest that FetA represents a potentially new target for developing therapeutics in the management of lipid-induced insulin resistance and type 2 diabetes. Insulin resistance (IR) in peripheral tissues, e.g., adipose tissue, skeletal and cardiac muscles and liver appears to play a major pathophysiologic role in the development of metabolic syndrome (MetS) and type 2 diabetes (T2D). While IR in muscles and adipose tissue accounts for around 80- 90% impairment of peripheral glucose uptake, toll-like receptor 4 (TLR4) plays a key role in innate immunity by activating an inflammatory signaling pathway. Free fatty acids (FFAs) stimulate adipose tissue inflammation through the TLR4 pathway, resulting in insulin resistance. However, current evidence suggests that FFAs do not directly bind to TLR4, but an endogenous ligand for TLR4 remains to be identified. Here we show that fetuin-A (FetA) could be this endogenous ligand and that it has a crucial role in regulating insulin sensitivity via Tlr4 signaling in mice. FetA (officially known as Ahsg) knockdown in mice with insulin resistance caused by a high-fat diet (HFD) resulted in downregulation of Tlr4-mediated inflammatory signaling in adipose tissue, whereas selective administration of FetA induced inflammatory signaling and insulin resistance. FFA-induced proinflammatory cytokine expression in adipocytes occurred only in the presence of both FetA and Tlr4; removing either of them prevented FFA-induced insulin resistance. We further found that FetA, through its terminal galactoside moiety, directly binds the residues of Leu100-Gly123 and Thr493-Thr516 in Tlr4. FFAs did not produce insulin resistance in adipocytes with mutated Tlr4 or galactoside-cleaved FetA. Taken together, our results suggest that FetA fulfills the requirement of an endogenous ligand for TLR4 through which lipids induce insulin resistance. This may position FetA as a new therapeutic target for managing insulin resistance and type 2 diabetes.

In the accompanying editorial, Jan Heinrichsdorff & Jerrold M Olefsky said "Pal et al. shed new light on this subject by demonstrating that FetA, a liver-derived circulating glycoprotein, serves as an adaptor protein that directly links SFAs to TLR4 activation. The new studies by Pal et al. go a long way toward resolving how SFAs can activate TLR4 signaling despite their inability to bind TLR4 itself. The authors showed that there are elevated concentrations of FetA in the serum of obese humans and mice with diabetes when compared to healthy humans or wild-type mice and that this is accompanied by elevated inflammatory cytokine expression. Knockdown of TLR4 or FetA in obese insulin- resistant mice dramatically improved glucose homeostasis as a result of reduced activation of TLR4-mediated proinflammatory signalling cascades. Most convincingly, in vivo SFA infusion led to the expected state of insulin resistance in control mice, but FetA-knockdown mice were protected from these effects. The authors' findings that FetA knockdown improved glucose homeostasis to a similar degree as TLR4 knockdown and that FetA was necessary for SFA-dependent inflammatory gene expression suggested an association between SFAs, FetA and Tlr4. Furthermore, Pal et al.2 showed that FetA directly binds TLR4, and they went on to identify the protein domains on TLR4 responsible for its interaction with

FetA. Thus, SFAs bind FetA, FetA binds TLR4, and SFAs are unable to promote TLR4-dependent proinflammatory effects in the absence of FetA. Therefore, the authors conclude that FetA functions as an adaptor protein that connects SFAs to Tlr4." [Heinrichsdorff J, Olefsky JM. Fetuin-A: the missing link in lipid-induced inflammation. Nat Med. 2012 Aug;18(8):1182-3. doi: 10.1038/nm.2869. PMID: 22869185].

Adipocyte Fetuin-A contributes to Macrophage Migration into Adipose tissue and Polarization of Macrophages. eJournal of Biological Chemistry, vol. 288, no. 39, pp. 28324-28330, September 27, 2013.

Macrophage infiltration into adipose tissue during obesity and their phenotypic conversion from anti-inflammatory M2 to proinflammatory M1 subtype significantly contributes to inflammation mediated insulin resistance; signaling molecule(s) for these events, however, remains poorly understood. We demonstrate here that excess lipid in the adipose tissue environment may trigger one such signal. Adipose tissue from obese diabetic db/db mice, HFD-fed mice, and obese diabetic patients showed significantly elevated fetuin-A (FetA) levels in respect to their controls; partially hepatectomized HFD diet mice did not show noticeable alteration, indicating adipose tissue to be the source of this alteration. In adipocytes, fatty acid induces FetA gene and protein expres sions, resulting in its copious release. We found that FetA could act as a chemoattractant for macrophages. To simulate lipid-induced inflammatory conditions when proinflammatory adipose tissue and macrophages create an altered microenvironment, we set up a transculture system of macrophages and adipocytes.

Adipose tissue inflammation has been increasingly recognized as the primary cause of obesity-induced insulin resistance. Accumulation of macrophages in adipose tissue contributes to obesity-induced inflam- mation. Polarity of adipose tissue macrophages (ATM) is inclined toward proinflammatory or classically activated M1 phenotype from anti-inflammatory or alternatively activated M2 subtype. Proinflammatory cytokines from M1, in turn, adversely affect insulin activity in target tissues. Three issues are necessary in the dynamics of adipose tissue inflammation: (i) lipid-induced proinflammatory status of adipose tissue; (ii) recruitment of M2 macrophages into adipose tissue; and (iii) pro-inflammatory polarization of M2 macrophages to M1 subtype. Monocyte chemoattractant protein-1 (MCP-1) has been implicated in migration of M2 macrophages into adipose tissue, a recent study shows that only about 40% macrophage infiltration occurs with MCP-1. This suggests the existence of other factor(s) for macrophage infiltration into adipose tissue. Based on our observations on the presence of FetA in adipose tissue, we thought that FetA could be the other chemokine in addition to MCP-1. In this study, we show that FetA increases macrophage migration into adipose tissue. We also demonstrate that FA induces FetA gene and protein expressions in adipocytes followed by its release, causing M2 to M1 polarization. Adipose tissue of db/db mice showed more than 3-fold higher FetA level in compar ison with their lean non-diabetic control. A significant increase of FetA occurred in adipose tissue of HFD-fed mice and obese diabetic subjects. Excess lipid in adipose tissue probably influences FetA level. To examine this, we incubated adipocytes from non-obese SD-fed mice with palmitate and found a dose-dependent increase of FetA release into the medium. Incubation of 3T3-L1 adipocytes with FA in the presence of [3H]leucine followed by radiolabeled FetA immunoprecipitation showed dose-dependent increase in FetA synthesis in response to FA, coinciding with elevated FetA mRNA expression. As in liver, FA-induced adipocyte FetA expression was also found to be NF-KB-dependent; FA failed to express FetA in NF-KB KO cells or in the presence of NF-KB translocation inhibitor. In the ChIP assay, FA increased NF-KB binding to adipocyte FetA promoter, effecting its activation; this was inhibited by NF-KB inhibitors.

FetA Affects Macrophage Migration into adipose tissue

When we observed significantly elevated FetA content in HFD mice, we thought that this excess FetA could originate from adipose tissue itself. Incubation of adipocytes with FA produced 3-fold increase in FetA release; the stromal vascular fraction could not produce FetA. We then performed experiments to examine whether FetA could act as a chemoattractant for macrophage infiltration to adipose tissue by using a Boyden chamber system. Both FetA and MCP-1 affected THP1 migration through 5-μm

pores of Boyden chambers. Most likely, in inflammatory adipose tis sue where ATM is a co-inhabitant, both FetA and MCP-1 may be released concomitantly and produce additional effects. Such a possibility is reflected from the results obtained with FetA and MCP-1 combination. We validated these results by observing whether the chemoattractive effect of pro-inflammatory cytokines, if any, share results produced by FetA. Immuno-depletion of TNF-alpha, IL-6, or IL-1 or direct addition of these did not alter FetA-stimulated macrophage migration. These findings show that the effect of FetA on macrophage movement is not influenced by other related factors.

FetA Induces Macrophage Polarization in adipose tissue
In a trans-culture system, THP1 macrophages were seeded on the bottom, whereas hAdp were cultured onto the membrane of Trans-well cell culture inserts, and FA was added to these cells. Addition FA to hAdp greatly enhanced FetA release into the medium. Excess release of FetA from hAdp in response to FA was associated with overexpression of M1 markers in THP1 macrophage TNFalpha, IL-6, and MCP-1, and significant decline of M2 markers PPAR-gamma and IL-10. Elevated FetA release due to FA also enhanced TNF-alpha and IL-6 expression in THP1 and RAW macrophages; suppression of NF-KB reduced the effect of FetA on macrophage polarization as this also subdued FetA secretion. FA-induced FetA augmented TNF-alpha and IL-6 but decreased Arg-1 mRNA expression, whereas in TLR4 KO cells, the effect of FA was not observed, suggesting the involvement of FA-FetA-TLR4 pathway in this process.

On direct incubation of RAW264.7 cells with FetA, proinflammatory cytokine expressions increased with a corresponding decrease in anti-inflammatory cytokines. FetA failed to elicit such responses in the presence of CLI-095, a TLR4 inhibitor. FACS analysis demonstrated that FetA could significantly alter macrophage polarity as the majority of macrophages were converted to CD11c+ cells, indicating M1 phenotype. Intriguingly, the influence of FetA on macrophage migration and polarization during adipose tissue inflammation appears to be independent of its source from the liver. FA could efficiently stimulate FetA synthesis and secretion from adipocytes. FA-induced FetA synthesis in adipocytes is similar to hepatocytes. FA activates NF-KB, which binds to FetA promoter that in turn upregulates FetA gene expression.

In conclusion, it may be stated that three new details have been obtained from this investigation: (i) FetA origin and secretion within adipose tissue microenvironment, (ii) contribution of FetA in macrophage infiltration, and (iii) contribution of FetA in macrophage polarization.

Fetuin-A: A novel hepatokine modulating cardiovascular and glycemicnoutcomes in metabolic syndrome. Endocr Pract. 2014; 20:1345-1351.

Fetuin-A is a novel hepatokine. The number of biologic roles attributed to fetuin-A has increased exponentially in the past decade. The objective of this review is to discuss the pathophysiology of fetuin-A action, its proinflammatory and anti-inflammatory attributes in different biological systems throughout the body, and pharmaco- logic interventions that modulate fetuin-A levels.

Fetuin-A is the endogenous ligand for Toll-like receptor-4 activation, for lipid-induced insulin resistance. Fetuin-A has inverse interaction with adiponectin. Increased fetuin-A is a risk factor for NAFLD and T2D. Fetuin-A is a negative acute-phase reactant in sepsis and endotoxemia, promotes wound healing, and is neuroprotective in Alzheimer's disease. Decreased fetuin-A predicts increased disease activity in Crohn's disease, COPD and ulcerative colitis. Both elevated and reduced fetuin-A may be linked with increased cardiovascular events.

Fetuin-A is a pleotropic molecule with diverse (sometimes even contradictory) effects in different systems, brought about by interaction with a variety of receptors, including the insulin, transforming growth factor-beta, and a plethora of Toll-like receptors. As a pro-in flammatory molecule, fetuin-A contributes to insulin resistance and is an important link between liver, adipose tissue and muscles. Fetuin-A is neuroprotective and plays an important anti-inflammatory role in sepsis and autoimmune

disorders. Pharmacologic options are limited in modulating serum fetuin-A but salsalates, curcumin, and vitamin D are promising agents to look for.

In contrast to the initial belief that fetuin-A is only a circulatory inhibitor of vascular calcification, it is now recognized as a pleotropic molecule associated with diverse—sometimes even contradictory—effects in different body systems. This is due to the ability of fetuin-A to interact with a variety of receptors. As a proinflammatory molecule, fetuin-A plays an important role in the genesis of IR and is an important molecular link between the liver, adipose tissue, and muscles. The relationship between fetuin-A, cardiovascular events, and autoimmune diseases is complex and yet to be fully elucidated.

Fetuin-A downregulates adiponectin through Wnt-PPARγ pathway in lipid induced inflamed adipocyte. Biochim Biophys Acta. 2016 Oct 6;1863(1):174-181.

Adiponectin, an adipokine, is an anti-diabetic and anti-atherogenic agent. Low adiponectin levels are seen commonly in obesity induced T2D. However, underlying mechanism of low adiponectin levels in obese diabetic condition is yet unclear. We observed low plasma adiponectin levels to coincide with high Fetuin A (FetA) levels in HFD induced obese diabetic mice. Knock down of FetA gene ($FetA^{KD}$) elevated adiponectin level markedly in HFD mice, while reinforcement of FetA into $FetA^{KD}$HFD mice reduced its level. These results indicate FetA's involvement in the lowering of adiponectin level in obesity induced diabetic mice. Our findings to understand how FetA could deplete adiponectin demonstrated that FetA could enhance Wnt3a expression in HFD mice adipocytes. Addition of FetA to 3T3L1 adipocyte increased Wnt3a expression in a dose dependent manner. Overexpression of Wnt3a by FetA inhibited PPARγ and adiponectin. FetA failed to reduce PPARγ and adiponectin in Wnt3a gene knocked down 3T3L1` adipocytes. All these suggest that FetA mediate its inhibitory effect on adiponectin through Wnt3a-PPARγ pathway. Inhibition of adiponectin expression through FetA and Wnt3a significantly compromised with the activation of AMPK and its downstream signalling molecules which adversely affected lipid management causing loss of insulin sensitivity. Downregulation of adiponectin in inflamed adipocyte by FetA through the mediation of Wnt3a and PPARγ is a new report.

Plasma fetuin-A triggers inflammatory changes in macrophages and adipocytes by acting as an adaptor protein between NEFA and TLR-4. Diabetologia. 2016 Apr;59(4):859-60.

We read with interest the research letter entitled 'Plasma fetuin-A does not correlate with mono- cyte TLR4 in humans' by Jialal and colleagues. The authors state that: 'Studies using animal models, largely the work of Pal et al, have elegantly demonstrated that fetuin-A is the endogenous ligand for TLR4 via which lipids such as fatty acids induce insulin resistance, but there are no data in support of this in humans. This is not, in fact, the case. In the paper referred to, we did provide data on human adipocytes from both diabetic and non-diabetic participants showing that fetuin-A acts as an endogenous ligand of TLR4 to induce insulin resistance. In another recent review, Stefan and Häring observed a significant correlation between circulating fetuin A and insulin resistance in humans.
Jialal et al also state: 'Based on our findings, we propose that until larger studies report significant correlations between TLR4 expression in monocytes/adipose tissue and circulating fetuin-A in humans with obesity, the metabolic syndrome or diabetes, the relationship between these two proteins demonstrated in animals might not translate to humans'. In our Nature Medicine paper, we have clearly shown a significant correlation between plasma fetuin-A and TLR4 expression in human adipocytes. We have shown that circulating NEFA stimulates the pro duction of fetuin-A by the liver via NF-κB. Fetuin-A then forms a dimer with NEFA by acting as its binding protein. The NEFA–fetuin-A dimer finally binds to TLR-4 present on the surface of adipocytes and macrophages, forming a ternary complex that triggers a local inflammatory response in the adipose tissue. Upon stimulation by circulating fetuin-A, white adipose tissue attracts circulating M2 macrophages that undergo proinflammatory polarisation to M1 under the influence of fetuin-A generated locally by the inflamed adipocytes. Jialal et al drew conclusions based on their studies on circulating monocytes, which are not

the most appropriate model to study the NEFA–fetuin-A–TLR-4 relationship. Such interactions only take place in the adipose tissue. Moreover, our hypothesis concerned TLR4 activation and NOT expression, which Jialal et al went on to study. Finally, their concluding remarks on fetuin-A and TLR4 expression in monocytes/ adipose tissue goes against the existing literature on the subject and creates a lot of confusion.

Palmitate induced Fetuin-A secretion from pancreatic β-cells adversely affects its function and elicits inflammation. Biochem Biophys Res Commun. 2017 Sep 30; 491(4):1118-1124.

Islets of type 2 diabetes patients display inflammation, elevated levels of cytokines and macrophages. The master regulator of inflammation in the islets is free fatty acids (FFA). FFA and TLR4 stimulation induces pro-inflammatory factors in the islets. We demonstrate that excess lipid triggers Fetuin-A (FetA) secretion from the pancreatic b-cells. Palmitate treatment to MIN6 cells showed significantly elevated FetA levels in respect to their controls. Fatty acid induces the FetA gene and protein expression in the pancreatic b-cells via TLR4 and over-expression of NF-kB. In the NF-kB knocked down MIN6 cells palmitate could not trigger FetA release into the incubation medium. These results suggest that NF-kB mediates palmitate stimulated FetA secretion from the pancreatic b-cells. Blocking the activity of TLR4 by CLI-095 incubation or TLR4 siRNA restored insulin secretion which confirmed the role of TLR4 in FFA-FetA mediated pancreatic b-cell dysfunction. Palmitate mediated expression of NF-kB enahanced inflammatory response through expression of cytokines such as IL-1b and IL-6. These results suggest that FFA mediated FetA secretion from pancreatic b-cells lead to their dysfunction via FFA-TLR4 pathway. FetA thus creates an inflammatory environment in the pancreatic islets that can become a possible cause behind pancreatic b-cell dysfunction in chronic hyperlipidemic condition.

A Small Insulino-mimetic Molecule Also Improves Insulin Sensitivity in Diabetic Mice. PLoS One. 2017 Jan 10;12(1): e0169809.

In this report, we describe a peroxyvanadate small molecule DmpzH[$VO(O_2)_2$(dmpz)], henceforth referred as dmp, which specifically binds to and activates insulin receptor tyrosine kinase and its downstream signaling molecules resulting increased uptake of [^{14}C] 2 Deoxy-glucose. Oral administration of dmp to streptozotocin treated BALB/ c mice lowers blood glucose level and markedly stimulates glucose and fatty acid uptake by skeletal muscle and adipose tissue respectively. In db/db mice, it greatly improves insulin sensitivity through abundant expression of PPARγ and its target genes i.e. adiponectin, CD36 and aP2. Study on the underlying mechanism demonstrated that excess expression of Wnt3a decreased PPARγ whereas dmp suppression of Wnt3a gene increased PPARγ expression which subsequently augmented adiponectin. Increased production of adiponectin in db/db mice due to dmp effected lowering of circulatory TG and FFA levels, activated AMPK in skeletal muscle and stimulated mitochondrial biogenesis and bioenergetics.

Impairment of energy sensors, SIRT1 and AMPK, in lipid induced inflamed adipocyte is regulated by Fetuin A. Cellular Signalling 42 (2018) 67–76.

Adipocyte dysfunction secondary to excess lipid accumulation leads to the disruption of cellular energy homeostasis; the underlying mechanism of this event remains unclear. We demonstrate that FetuinA (FetA) plays a critical role in the impairment of SIRT1 and AMPK in inflamed adipocytes of high fat diet (HFD) mice. A linear increase in adipocyte hypertrophy from 10 to 16 week was in tandem with the increase in FetA and coincided with SIRT1 cleavage and decrease in pAMPK, adversely affecting PGC1α activation. Knock down (KD) of FetA gene in HFD mice significantly improved this situation indicating FetA's contribution in the damage of energy sensors in inflamed adipocyte. This FetA effect was mediated throughTNF-α which again is dependent on FetA, as the latter augments the expression of the former. Suppression of FetA prevented TNF-α mediated Caspase-1 activation and cleavage of

SIRT1. FetA induced inactivation of PGC1α due to SIRT1 cleavage, decreased PPARϒ, adiponectin, NRF1 and Tfam expression. All of these events together caused a significant fall of in mitochondrial biogenesis and bioenergetics that disrupted energy homeostasis resulting in loss of insulin sensitivity. Taken together, our findings revealed a new dimension of FetA, it not only induced inflammation in adipocyte but also acts as an upstream regulator of SIRT1 cleavage and AMPK activation. Intervention of FetA may be worthwhile to prevent metabolic imbalance that causes insulin resistance and type 2 diabetes.

Increase in PPARγ _inhibitory phosphorylation by Fetuin—A through the activation of Ras-MEK-ERK pathway causes insulin resistance. BBA - Molecular Basis of Disease 1867 (2021) 166050.

Obesity induced insulin resistance is primarily regulated by inhibitory phosphorylation of PPAR-γ at serine 273 (PPARγS273), previously shown to be regulated by MEK and ERK. Here we analyzed the involvement of Fetuin-A (FetA) as an upstream regulator molecule for the regulation of PPARγS273. Mice fed with standard diet (SD), high fat diet (HFD) and HFD with FetA knockdown (HFD-FetAKD) were used to examine the role of FetA on PPARγS273 phosphorylation in adipocytes. The mechanism of regulation and its effect on skeletal muscle were studied using primary adipocytes, 3T3-L1 and C2C12 cell lines. Increased FetA in HFD mice strongly correlated with augmentation of PPARγS273 phosphorylation in inflamed adipocytes while knockdown of FetA suppressed it. This effect of FetA was mediated through the activation of Ras which in turn activated MEK and ERK. On addressing how FetA could stimulate activation of Ras, we found FetA to trigger TNFα in inflamed adipocytes. The ensuing sharp fall in adiponectin level attenuated AMPK activation in skeletal muscle affecting mitochondrial ATP production. Our data reveal the essential role of FetA induced Ras activation in regulating PPARγ inhibitory phosphorylation through Ras-MEK-ERK pathway which downregulates adiponectin and thereby mitochondrial bioenergetics.

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