Published in final edited form as:

Immunogenetics. 2013 January ; 65(1): 83–90. doi:10.1007/s00251-012-0659-4.

Influence of Tyrphostin AG490 on the expression of diabetes associated markers in human dipocytes

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Abstract

Tyrosine kinase inhibitors (TKi) hold promise as a treatment for a variety of disorders ranging from those in oncology to diseases thought immune mediated. Tyrphostin AG490 is a potent Jak-Stat TKi shown effective in the prevention of allograft transplant rejection, experimental autoimmune disease, as well as the treatment of cancer. However, given its ability to modulate this important but pleiotropic intracellular pathway, we thought it is important to examine its effects on glucose metabolism and expression of major transcription factors and adipokines associated with insulin insensitivity and diabetes. We investigated the metabolic effects of AG490 on glucose levels *in vivo* using an animal model of diabetes, non-obese diabetic (NOD) mice, and transcription factor expression through assessment of human adipocytes. AG490 treatment of young non-diabetic NOD mice significantly reduced blood glucose levels ($p=0.002$). *In vitro*, treatment of adipocytes with rosiglitazone, an insulin sensitizer that binds to PPAR receptors and increases the adipocyte response to insulin, significantly increased the expression of the anti-diabetic adipokine adiponectin. Importantly, the combination of rosiglitazone plus Tyrphostin AG490 further increased this effect, and was specifically associated with significant upregulation of C/EBP ($p<0.0001$). In terms of the mechanism underlying this action, regulatory regions of the *PPAR γ* , *ADIPOQ* and *C/EBP* contain the Stat5 DNA binding sequences and were demonstrated, by gel shift experiments *in vitro*. These data suggest that blocking Jak-Stat signaling with AG490 reduces blood glucose levels and modulates the expression of transcription factors previously associated with diabetes, thereby supporting its potential as a therapy for this disease.

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Disclosure

Authors declare no conflict of interest.

Keywords

Adipocytes; Diabetes; Kinase Inhibitor; Autoimmunity; AG490; Obesity

Tyrosine kinase inhibitors (TKi) represent a promising class of drugs for the treatment of disease. Amongst those most promising and advanced are those seeking to prevent transplant rejection (Behbod et al. 2001; Changelian et al. 2003; Higuchi et al. 2005; Saemann et al. 2004; Stepkowski et al. 2001; Stepkowski et al. 2002) and providing an efficacious treatment for a variety of cancers (Baselga 2006; Hagerkvist et al. 2007; Santos and Verstovsek 2011). This hope has also recently been extended to the field of autoimmune disease where promising preclinical data have been observed in mouse models of multiple sclerosis (Crespo et al. 2011), intestinal inflammation (Sidhu et al. 2011), experimental autoimmune encephalomyelitis (EAE) (Bright et al. 1999)(Bright et al. 1999; Constantin et al. 1998; Constantin et al. 1999), and type 1 diabetes (T1D) (Louvet et al. 2008; Mokhtari and Welsh 2010). Indeed, with respect to this latter disorder, oral administration of Sunitinib (Sutent) and Imatinib (Gleevec), two TKi, have been shown to prevent as well as reverse T1D in NOD mice (Louvet et al. 2008). Tyrphostin AG 490, another form of TKi, is a potent inhibitor of the Jak-Stat signaling pathway (Goncalves et al. 2010) and reportedly, more active as a Jak2 inhibitor than Jak3 (Luo and Laaja 2004). Additionally, Tyrphostin AG490 has no impact on membrane associated kinases such as Lyn, Btk, Syk, Src and Zap70 (Kirken et al. 1999; Meydan et al. 1996), suggesting that it is not a general kinase inhibitor (Kirken et al. 1999; Luo and Laaja 2004; O'Shea 1997; O'Shea et al. 2004; Wang et al. 1999). With respect to specific effects of Tyrphostin AG 490 on the immune response, it has been noted to block IL-12 mediated T-cell proliferation, inhibit phosphorylation of Stat3, decrease IFN- γ production mediated by IL-12 (Bright et al. 1999), and inhibit the differentiation of antigen-specific Th1 cells *in vivo* (Changelian et al. 2003). Recently, we demonstrated that *in vivo* administration of Tyrphostin AG 490 prevents and reverses T1D in NOD mouse (Davoodi-Semiromi et al. 2012a; Davoodi-Semiromi et al. 2012b), suggesting that this drug possess immunomodulating properties; however, potential metabolic effects (i.e., those separate of immunological in nature) of this compound have not been described. Compelling evidence demonstrates that the Jak-Stat signaling pathway plays a critical role in adipocyte development and function (Baugh, Jr. et al. 2007; Coulter and Stephens 2006; Floyd et al. 2007; Richard and Stephens 2011; Stewart et al. 2011; White et al. 2007; White and Stephens 2010a; White and Stephens 2010b; Zhang et al. 2011). Therefore, we investigated the effects of Tyrphostin AG 490 on glucose levels *in vivo* and on several major transcription factors associated with diabetes; namely PPAR γ 1 (peroxisome proliferator activated receptor gamma 1), PPAR γ 2, C/EBP (C-enhanced binding protein), AP-2 (activating protein- 2), adiponectin and two members of the STAT family; Stat5 and Stat3. These assessments were performed both *in vitro* via mRNA analysis as well as at a protein level; both involving human adipocytes. The resulting data suggest that Tyrphostin AG 490 regulates glucose metabolism *in vivo* and modifies the molecular activation of diabetes related transcription genes favoring increased insulin sensitivity, leading to what would be considered improved diabetes status. Our data also suggest the Jak-Stat signaling pathway negatively regulates gene expression of key factors playing a vital role in glucose metabolism.

With respect to our experimental plan, female NOD/LtJ were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in ventilated cages in a specific pathogen free facility at the University of Florida. All mice were maintained and handled in accordance with the University of Florida Institutional Animal Care and Use Committee (IACUC). Tyrphostin AG 490 (EMD Chemicals, Gibbstown, NJ), 5 mg, was initially dissolved in sterile DMSO (EMD Chemicals) and brought to final volume by sterile PBS. One vial of compound containing 5 mg of AG490 was injected into 5 mice (1 mg/mouse) via intraperitoneal (i.p) route. Mice (4 weeks of age) were treated three times per week for 5 consecutive weeks and were euthanized one week after the last injection at week 10. Concentration of Tyrphostin AG 490 in all *in vitro* studies was 10 μ M. Rosiglitazone was purchased from Cayman Chemical (San Francisco, CA) and a final concentration of 2 μ M used in all experiments described herein. SGBS (Simpson-Golabi-Behmel-Syndrome) cell culture and adipocyte differentiation were performed as described elsewhere (Fischer-Posovszky et al. 2008; Wabitsch et al. 2001). Fully differentiated adipocytes were starved from 3FC medium for 1–3 hours, and then left either untreated (control), treated with rosiglitazone alone, or with Tyrphostin AG 490 plus rosiglitazone for 0.5, 2 and 16 hours (overnight) under 3FC conditions.

Real-Time TaqMan Gene expression analyses was performed on total RNA extracted from SGBS cell cultures using RNAeasy kits (QIAGEN) and converted to cDNA using High Capacity cDNA Reverse Transcription kits (Applied Biosystems) followed by PCR amplification using a 7300 Applied Biosystems PCR machine. Electrophoresis mobility shift assays (EMSAs) were performed as described elsewhere (Davoodi-Semiromi et al. 2004; Davoodi-Semiromi et al. 2007a). All analyses for statistical significance were performed using Prism 5.0 software (GraphPad Software Inc, La Jolla, CA). Real-Time data [ddct (Relative Quantitation)] was analyzed using integrated SDS software, V1.4.0 (Applied Biosystems) and RQ value (fold change) as described elsewhere (Davoodi-Semiromi and Cooper-Dehoff 2011) with *p* value less than 0.05 considered statistically significant.

Prediabetic NOD mice were treated either with Tyrphostin AG490 (1 mg/mouse) or vehicle (DMSO + PBS) three times per week for 5 consecutive weeks and body weight and blood glucose level was measured at least 1–2/week. As shown in figure 1A and B (n=15/group), i.p. administration of Tyrphostin AG490 had no effect on body weight gain or loss; however, blood glucose values were significantly lower in Tyrphostin AG490 treated NOD mice at week 8 (*p*=0.0021, n=15, figure 1) when compared with DMSO treated mice (n=14), suggesting metabolic effects of this compound on glucose metabolism. As noted previously, we recently demonstrated successful prevention and reversal of T1D in NOD mice (Davoodi-Semiromi et al. 2012a; Davoodi-Semiromi et al. 2007b), opening the possibility that the Jak-Stat signaling pathway has a negative effect on glucose metabolism in situations of ongoing inflammation; yet, this notion required investigation.

An additive and significant upregulation of PPAR γ 2, but not PPAR γ 1 at both an mRNA and protein level was observed when SGBS were treated with rosiglitazone and Tyrphostin AG490 (see figure 2B and D); however, this effect was quenched when the cells were treated for a longer period of time (16 hours). Sustained upregulation of PPAR γ at protein level was observed by rosiglitazone when the cells were treated for 16 hours (figure 2D) and

combination of rosiglitazone plus Tyrphostin AG490 slightly increased expression of $PPAR\gamma 2$ when compared to the control. This data suggests that $PPAR\gamma 2$ is an early gene responding gene and second, that the Jak-Stat signaling pathway negatively regulates expression of $PPAR\gamma 2$ but not $PPAR\gamma 1$. As shown in figure 3, SGBS cells treated with rosiglitazone alone, or a combination of rosiglitazone plus Tyrphostin AG490 had no effect on expression of *C/EBP* or the anti-diabetic adipokine, adiponectin, within 2 hours; however, significant upregulation of adiponectin was observed when SGBS cells were treated either with rosiglitazone or with a combination of rosiglitazone plus Tyrphostin AG490. Furthermore, this was associated with marked upregulation of *C/EBP* for a longer period of time (see figure 3 right panel). We also examined the effects of these drugs on *AP2* gene expression at different time points, with no significant differences observed (data not shown); suggesting that upregulation of adiponectin by rosiglitazone and Tyrphostin AG490 is not mediated via this transcription factor. Since insulin receptors play a major role in diabetes, we also examined the effects of these two drugs, by real-time RT-PCR, on the expression of insulin receptors at different time points. The combination of rosiglitazone plus Tyrphostin AG490 slightly increased insulin receptor expression, albeit this did not reach statistical significance (data not shown). Taken together, these data suggest that rosiglitazone treatment alone and/or in combination with Tyrphostin AG490 ameliorates expression of adiponectin, and is likely mediated via the *C/EBP* but not through *PPAR\gamma 1-2*. Furthermore, the Jak-Stat signaling pathway negatively regulates expression of adiponectin. Additionally, our data suggest that the *PPAR\gamma 1* and *PPAR\gamma 2* genes responded very quickly to stimuli and thus, can be considered as early responding genes. However, the other two genes (i.e., *AP2* and *C/EBP*) are late responding genes given their response patterns to stimuli (supplementary Table 1).

We reasoned if expression of *PPAR \gamma*, *ADIPOQ* and *C/EBP* were regulated by the Jak-Stat signaling pathway, the Stat DNA binding consensus sequences should be present within the regulatory region of these genes. As shown in figure 4A and supplementary table 1, the Stat5 DNA binding sequences were found within the regulatory region of *C/EBP*, *PPAR\gamma* and *ADIPOQ*, respectively. This data suggests that these genes are downstream to the Jak-Stat5. Furthermore, as shown in figure 4B, activated Stat5 bound to oligonucleotides containing the Stat5 consensus sequences [TTG (n)³ CAA] in EMSAs experiments. The Stat5/DNA complexes were observed at expected position and by application of anti-Stat5 antibody (lane 4), the Stat5/DNA complexes were shifted to higher position (figure 4b, lane 4; supershift) suggesting that the Stat5 contributes in the DNA/protein complex. Nonetheless, addition of anti-Stat5 antibody was unable to lift the entire protein/DNA complex to the supershift position, suggesting that other STAT family members and/or other protein(s) may contribute to formation of this complex. With this observation, further *in vitro* and *in vivo* studies are warranted to investigate which of the multiple Stat5 DNA binding sequences are functionally active.

The STAT family members including Stat3 and Stat5 play crucial roles in differentiation and development of many cell types including those related to adipocyte development. We previously reported the effects of Tyrphostin AG490 on activation of Stat5 (Murawski et al. 2006). As shown in figure 5, rosiglitazone treatment and/or in combination with Tyrphostin

AG490 markedly decreased phosphorylation of Stat5 in adipocytes, with no effect on total Stat5 protein when cells were treated for 30 minutes (the first two top panels: comparison of lanes 2 and 3 versus lane 1). With these observations, one could consider that an anti-diabetic property of rosiglitazone is at least in part due to inactivation of the Jak-Stat5 signaling pathway. Additionally, rosiglitazone increased activation of the truncated Stat5 when the cells were treated for 30 minutes (figure 5, top panel, lane 2 versus lane 1), further blocking activation of the Jak-Stat pathway. Truncated Stat5 bound strongly with the DNA (see figure 4B); however, it would not be able to initiate the process of transcription due to the absence of transcription domain (i.e., a dominant negative effect). As expected, addition of Tyrphostin AG490 markedly blocked phosphorylation of Stat5 (figure 5, top panel, lane 3 versus lane 1) and an additive effect was observed when Tyrphostin AG490 was combined with rosiglitazone (figure 5, top panel, lane 2 versus lane 3). Total Stat5 protein did not change when the cells were treated for 30 minutes; however, combination of rosiglitazone and Tyrphostin AG490 for longer periods of time had no effect on total Stat3 protein, but did change total Stat5 protein levels. Based on our data shown in figure 5, longer treatment of adipocytes (i.e., 16 hours) with rosiglitazone and/or in combination with Tyrphostin AG490 ameliorates the truncated form of the activated Stat3 similar to what was observed for the Stat5 (see figure 5 top panel). The phosphorylated form of the Stat3 was very low under conditions used in our experiments and drug treatment had little or no effect on total Stat3 gene expression.

The Jak-Stat signaling pathway is the most important signaling pathway for transduction of signals from the cell surface to the nucleus (Ghoreschi et al. 2009; Ihle 1996; Imada and Leonard 2000; O'Shea et al. 2005). Based on our observations and those of others, we believe this signaling pathway has more roles in the pathogenesis and/or treatment of autoimmune diseases, transplantation and cancer than currently appreciated. Our studies also suggest that the prevention and reversal of T1D observed in our recent studies involving Tyrphostin AG490 treatment of NOD mice was achieved via regulation of both metabolic and immune parameters. (Davoodi-Semiromi et al. 2012a; Davoodi-Semiromi et al. 2012b).

The presence of the multiple Stat5 DNA binding consensus sequences within regulatory region of *PPAR γ* , *ADIPQ* and *C/EBP* also appear clinically relevant. Two studies demonstrated that administration of troglitazone, a *PPAR γ* agonist, significantly delayed the onset of T1D in NOD mice (Augstein et al. 2003; Beales et al. 1998); an outcome in agreement with our data presented here. Additionally, differential expression of *PPAR α* , *PPAR γ 1* and *PPAR γ 2* in different immune cell populations in NOD mice have also been reported (Yaacob et al. 2009). In this study, the expression of *PPAR γ 2* was impaired in macrophages and in CD4 T-cells of NOD mouse at three different stages of diabetes development (Yaacob et al. 2009). Additionally, these investigators showed that expression of *PPAR α* was significantly lower in peritoneal macrophages in diabetic mice (Yaacob et al. 2009), again in agreement with our data suggesting a crucial role of the PPAR family in pathogenesis of diabetes. The presence of multiple Stat DNA binding consensus sequences within the regulatory regions of three important genes related to diabetes development suggests cross talk exists between the Jak-Stat signaling pathway and these genes. However, understanding genetic interactions among the Jak-Stat signaling pathway and PPAR family,

along with adiponectin appears of importance for future investigations. The application of rosiglitazone alone and in combination with Tyrphostin AG490 markedly blocked phosphorylation of Stat5 and increased the truncated form of this important transcription factor (see figure 5), thereby further inhibiting the transcription machinery of this protein (Davoodi-Semiromi et al. 2007a). Furthermore, we have demonstrated that at mRNA and protein level inactivation of the Jak-Stat signaling pathway by Tyrphostin AG490 possesses anti-diabetic properties and this is at least achieved via upregulation of transcription factor *C/EBP* and adiponectin which is known to have anti-diabetic properties. Our gel-shift experiments further support this notion that these genes can actually be regulated by the Jak-Stat pathway; however, replication of the same data using total or nuclear protein extracted from adipocytes or adipose tissues awaits to be seen.

Taken together, the presence of the Stat5 DNA binding consensus sequences within the promoter region of three important diabetes associated factors further emphasize the potential importance of the Jak-Stat signaling pathway in pathogenesis of diabetes. In addition, appropriate blocking of this signaling pathway may have broad implications for prevention and/or treatment of several diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by grants from the Juvenile Diabetes Research Foundation International (Innovative grant # 5-2006-937), and NIH R21A176394-01 (The American Recovery And Reinvestment Act) awarded to ADS.

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Figure 1. Administration of Tyrphostin AG 490 reduces blood glucose level in pre-diabetic NOD mice

Female NOD mice (n=15) were injected (i.p) at 4 weeks of age with Tyrphostin AG490 (1mg/mouse), while control animals received the same number of injections of the vehicle (DMSO and PBS), three times per week. Blood glucose levels were measured at least twice per week. Blood glucose levels and body weight of Tyrphostin AG490 and DMSO treated mice at week 8 are shown.

Figure 2. Tyrophostin AG 490 treatment increased expression of PPARγ in SGBS

Fully differentiated SGBS were left untreated (control) and/or were treated with rosiglitazone alone or combination of rosiglitazone plus Tyrophostin AG490 for various periods of time (A–C). Data shown represent the mean of RQ values of two independent cell culture experiments, with two RT-PCR. D- Fully differentiated SGBS cells were left untreated or treated with rosiglitazone alone and/or with a combination of rosiglitazone plus Tyrophostin AG490. The effects of drug treatment on gene expression of two different isoforms of PPARγ at protein level are shown.

Figure 3. Tyrphostin AG490 treatment increases expression of adiponectin and this is associated with upregulation of C/EBP in SGBS

Differentiated adipocytes were left untreated (control) and/or were treated with rosiglitazone alone or with a combination of rosiglitazone plus AG490 for various periods of time. Real-time TaqMan gene expression of two independent cell culture experiments and at least two independent real-time experimental data sets are shown.

Figure 4. PPAR γ , ADIPOQ and C/EBP are putative Jak-Stat target genes

A) Approximately 20 Kb upstream of the start coding sequence (ATG) in PPAR γ , ADIPOQ and C/EBP were searched for the presence of the Stat5 DNA binding consensus sequence [TTC(N³)AA]. The position of each sequence from the start codon in the positive/negative strand is shown. (B) Total splenocyte protein of B6 mice were hybridized with double stranded oligonucleotides containing the Stat5B DNA binding sequences (5'-ACTGT**TTC**tttGAAGCTGT-3'). The core sequences are shown underlined and in bold. Complementary oligonucleotide were used to make double stranded DNA, were end-labeled, and used in EMSAs experiments as described in the materials and methods. Lane 1: no protein, probe only, lane 2: unactivated Stat5, lane 3- activated Stat5 (shift complexes are shown in white squares), Lane 4: activated Stat5 (supershifted complexes are shown in

white rectangles). This experiment represents one of at least three independent experiments with consistent results. Ab: antibody; Trn: truncated; NS: non-specific; FP: free-probe. Note: These figures (figure 4A and B) are intended to be in color online and black and white in print.

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Figure 5A

Figure 5. The effects of rosiglitazone and Tyrphostin AG 490 on phosphorylation of STAT3 and STAT5 in SGBS

(A) Differentiated adipocytes were left untreated (control) and/or were treated with rosiglitazone and or a combination of rosiglitazone plus Tyrphostin AG490 for 0.5 hr and 16 hours (overnight). Ten micrograms of total protein was loaded onto 10% acrylamide SDS-gel and the membrane bound protein was initially hybridized with anti-phosphorylated antibody and then re-hybridized with anti-pan antibody of the same protein. The membrane finally was hybridized with anti-actin antibody to prove equal protein loading in each well. (B) Densitometry of activated Stat5 (full-length) and total Stat5 was measured and normalized based on the control. This represents one of two independent experiments with the same results. Rosi = rosiglitazone; FL = full length; Trn = truncated.