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the Metabolic Transcriptional Coactivator PGC1-alpha

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Title:

Maintenance of Glucose Homeostasis Through Acetylation of the Metabolic Transcriptional Coactivator PGC-1 α .

Abstract (160 words)

The main purpose of this proposal is to test the hypothesis that acetylation of PGC-1 α by GCN5 and associated proteins, Pc3 and WDR18, controls hepatic glucose production. The major findings of this Research Technical Report are in tasks 2, 4, 5 and 6. In task 2, we have further confirmed that Pc3 and WDR18 are part of the PGC-1 α /GCN5 complex but are not required for its assembly. In task 4, we have analyzed the effects of Pc3 and WDR18 on gluconeogenic/glycolytic genes. In task 5, we have analyzed the negative effects of GCN5 on hepatic glucose metabolism. In Task 6, we have generated GCN5 and PGC-1 α shRNA adenoviruses to knock-down these genes in liver. Overall, the experiments reported indicate that PGC-1 α acetylation is a key chemical switch that in response to fed/fasting controls liver metabolism. We will continue to complete the proposed tasks to understand how PGC-1 α acetylation controls hepatic glucose production through the GCN5 complex.

Introduction

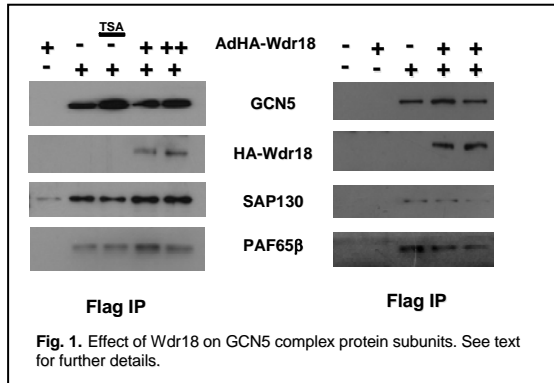
Homeostatic mechanisms in mammals, including humans, function to maintain blood glucose levels within a narrow range in response to hormones and nutrients. For example, high stress and intense exercise conditions combined with food deprivation make soldiers very vulnerable to changes in blood glucose levels. Glucose homeostasis is dysregulated in metabolic diseases such as obesity and diabetes which have high increased incidence in the US population. We study a biochemical process that controls blood glucose levels through control of hepatic glucose synthesis. This regulatory control is achieved by a chemical modification –acetylation- of the PGC-1 α metabolic transcriptional coactivator (Rodgers et al. 2005) (Lerin et al. 2006) (Rodgers and Puigserver, 2007). The overall purpose and scope of this Research Proposal is to decipher how two proteins, Pc3 and WDR18, that control the enzymatic activity of the PGC-1 α GCN5 Acetyl Transferase regulate PGC-1 α acetylation and its effects on glucose metabolism. We are using biochemical and physiological approaches, both in cell culture and mouse models, to precisely identify the key acetylation sites on PGC-1 α that are required and sufficient to modulate blood glucose levels. These findings have strong implications for the basic pathways of energy homeostasis, diabetes and metabolic diseases and will certainly benefit performance of personnel in the army that work in conditions of high stress. It is important to highlight that PGC-1 α chemical acetylation is directly controlled by two enzymes: GCN5 and SIRT1; this strengthens the possibility to use small molecules to target the catalytic activity of these proteins to manipulate PGC-1 α acetylation and normalize high glucose levels in diabetic patients.

Body

In this body section of this Research Technical Report, we will describe in detail the experimental data and interpretations of the results obtained in the third year of the award. As proposed tasks 1 and 3 were completed. In the third year we have performed experiments in tasks 2 and 4 (now completed), and 5 and 6. We have mainly accomplished the goals that were originally proposed and based on these results, we will continue the Tasks that were aimed to perform.

Task 2. Identification of the mechanisms of PGC-1 α 's repression by acetylation and interactions with GCN5-associated proteins Pc3 and WDR18 (Months 12-36).

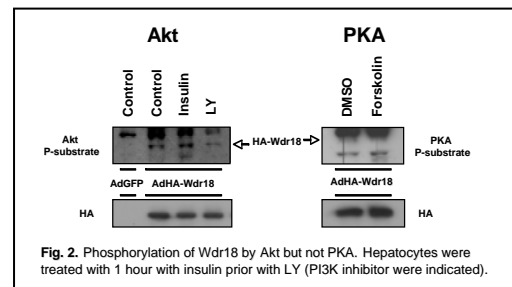
- *Analysis of physical interaction between PGC-1 α , transcriptional regulatory proteins that are known to control gluconeogenic genes and two novel proteins in the GCN5 protein complex (Pc3 and WDR18). (Months 12-36).*



were further confirmed with immunoblots using antibodies against two specific proteins of the GCN5 complex SAP130 and PAF65 β . As shown in Fig. 1, the expression of Wdr18 did not affect the interaction of two subunits of the GCN5 complex.

Together, this data will suggest that the effects of Wdr18 on PGC-1 α acetylation are not through alterations in the GCN5 complex subunits. Thus, it would seem that Wdr18 directly affects the enzymatic activity of GCN5.

In last year report, we showed that Wdr18 nuclear translocation depends on insulin signaling. This would fit with our current model, in high nutrient conditions PGC-1 α is acetylated through GCN5 acetyltransferase activity, however under low nutrient conditions Sirt1 deacetylase will keep PGC-1 α de-acetylated in an active form. In order to confirm whether Wdr18 was phosphorylated under insulin-stimulated conditions, we performed experiments using antibodies directed against an Akt substrate –Akt is an insulin activated kinase that is thought it transduces a large portion of insulin action-. As shown in Fig. 2, hepatocytes treated with insulin-induced phosphorylation of Wdr18. Interestingly there is one putative akt site in Wdr18 (Serine at position 254). In the next funded year we will investigate whether this specific site is responsible for insulin-induced translocation of Wdr18 that will provide a plausible regulatory control for GCN5-induced PGC-1 α acetylation in high nutrient conditions.



- *Chromatin Immunoprecipitation Analysis. Determination of PGC-1 α chromatin occupancy on gluconeogenic genes depending on GCN5 activation modulated by association with Pc3 and WDR-18 (Months 18-36).*

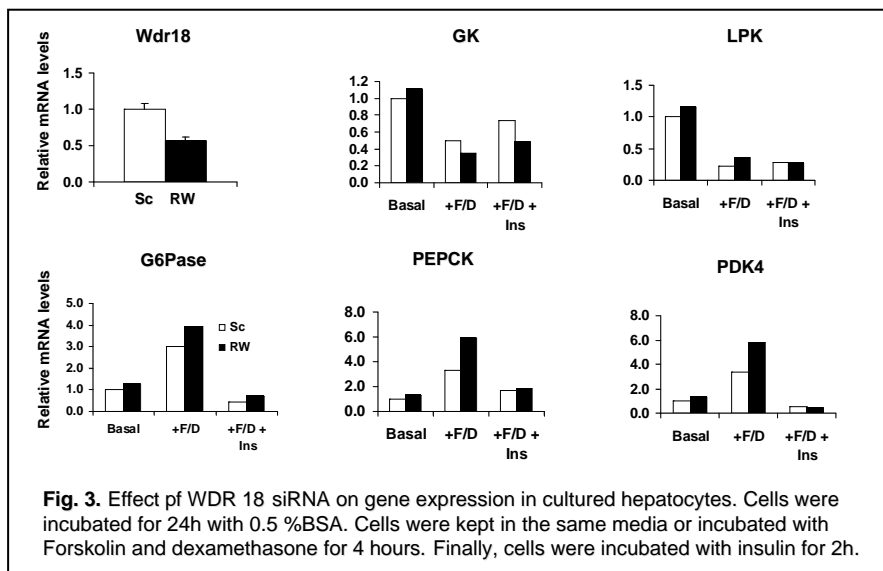
Our previous results on Chromatin Immunoprecipitation analysis have showed that PGC-1 α is recruited to promoter regions of gluconeogenic genes (Puigserver et al. 2003). More recently, we have also showed that GCN5 inhibited recruitment of PGC-1 α to these promoter through acetylation. Acetylated PGC-1 α was localized to nuclear speckles that were transcriptionally inactive (Lerin et al. 2006). The fact that Wdr18 increased PGC-1 α acetylation suggested that it

might alter recruitment of PGC-1 α to gluconeogenic promoters. In this year period we have tried several times to determine whether Wdr18 decreased PGC-1 α bound to chromatin of PEPCK and Glucose-6-Phosphatase promoters. So far the results have been negative (data not shown). Using Chromatin Immunoprecipitation analysis, we have tried the effects of Wdr18 on PGC-1 α bound to promoter of gluconeogenic genes in the presence or absence of exogenous GCN5. In the presence of GCN5 and as expected PGC-1 α was re-localized from the chromatin, however Wdr18 had a minor effect. In the next funded period, we will try again these experiments using Wdr18 siRNA; the prediction is that the depletion of Wdr18 would result in higher amounts of PGC-1 α bound to chromatin. Additionally, we will also perform experiments titrating the amounts of Wdr18 as well as GCN5. The idea behind this titration is that perhaps the experiments performed using overexpression of both proteins did not provide the proper stoichiometry.

Task 4. Identification of the mechanisms of PGC-1 α 's repression by acetylation and interaction with GCN5-associated proteins Pc3 and WDR-18. (Months 12-36).

- *Gene expression analysis by Northern Blot of gluconeogenic and glycolytic genes in cultured hepatocytes. Requirements of Pc3 and WDR18 by using specific siRNAs (Months 24-27).*

In order to test whether Pc3 and Wdr18 affect expression of genes involved in gluconeogenesis and glycolysis, we used specific shRNAs directed against both mRNAs. In last year report we



showed that we have generated specific shRNAs for Pc3 and Wdr18. As it relates to Pc3, we are still in the process of making adenoviral particles and we had some technical difficulties generating high titers. We plan in the next months of this year funded period to generate adenovirus for Pc3 with high titers in order to determine whether it controls expression of genes involved in hepatic

glucose metabolism.

We have successfully generated adenoviral particles at high titer to use in cultured hepatocytes to analyze expression of the genes mentioned above. Since we don't currently have an available antibody against Wdr18, we have validated the shRNA determining the effects on endogenous mRNAs levels (although not all shRNAs decrease levels of target mRNAs, a large percentage of designed shRNA indeed produce a decrease in mRNA) or by using transfected protein (shRNAs were designed against the coding region). As shown in Fig. 3 we have infected cultured hepatocytes with control shRNAs adenoviruses encoding an scrambled shRNA or adenoviruses

encoding for shRNA Wdr18. As expected, Wdr18 depletion decreases its own endogenous mRNA levels. We mimicked the fed/fasting response by using insulin (fed) and forskolin and dexamethasone (that induce cAMP and glucocorticoid receptor, fasting pathways). In the case of glycolytic genes we measured glucokinase (GK) and liver pyruvate kinase (LPK), two key genes in glycolysis that are strongly regulated in fed/fasting responses. In all conditions tested depletion of Wdr18 did not affect expression of GK or LPK (Fig. 3). As a control we show that under forskolin/dexamethasone treatment expression of both genes as strongly decreased as expected. We next tested the effect of Wdr18 shRNA on expression of gluconeogenic genes Glucose-6-Pase (G-6-Pase) and PEPCK, under basal conditions we did not detect any effect on these mRNAs, however under forskolin/dexamethasone treatment expression of both genes was significantly increased compared to control shRNA. Additionally, expression of PDK4 that is also induced in the fasting and is a target of PGC-1 α , displayed a similar expression pattern as gluconeogenic genes. Taken together, these results indicate that depletion of Wdr18 in hepatocytes increase expression of gluconeogenic genes but did not affect expression of glycolytic genes. These results would be consistent with our hypothesis that Wdr18 activates PGC-1 α acetylation through GCN5, therefore the lack of Wdr18 results in decreased GCN5 activity. In fact, the effects of Wdr18 depletion are similar to the effects reported using shRNA GCN5 (Lerin et al. 2006).

- *Glucose production analysis. Glucose measurement determinations from hepatocyte's – infected with adenoviruses encoding PGC-1 α acetylation mutants- culture medium. Requirement of Pc3 and WDR18 by using specific siRNAs (Months 24-30).*

In the past funded years we have generated all the reagents required to perform the experiments proposed in this task. We have generated adenoviruses encoding the PGC-1 α acetylation mutant (R13) –see task 5-; adenoviruses encoding Pc3 and WDR18. However, we have had technical problems generating a high titer of Pc3 to perform these studies –see above-. We have therefore decided to wait for this reagent in order to complete the last part of this task. It is expected that in the next months we will generate this reagent and proceed to perform the proposed experiments. Based on previous experiments and our hypothesis it is expected that the PGC-1 α acetylation mutant will be more active on secreting glucose, and Pc3 –positively- and Wdr18 –negatively- will affect PGC-1 α wild type but not mutant.

Task 5. Analysis of PGC-1 α acetylation mutants and requirement of GCN5 to control gluconeogenic/glycolytic fatty acid oxidation and mitochondrial gene expression in the liver of obese/diabetic mice. (Months 30-48).

- *Analysis of GCN5 requirement on the hepatic gluconeogenic/glycolytic, fatty acid oxidation and mitochondrial gene expression in the liver of obese/diabetic mice. Use of mice injected with adenoviruses encoding GCN5 siRNA. (Months 30-48).*

We have started to generate GCN5 siRNA directed against the mouse GCN5 mRNA. We have used in the past GCN5 shRNAs that were highly efficient to knock-down human or rat GCN5, but we have had technical problems to generate a mouse GCN5 shRNA. We are in the process of testing different sequences that are provided by Dharmacon and five different shRNAs that were generated in William Hanh's laboratory (Dana-Farber Cancer Institute). The strategic plan is to have this reagent successfully generated in the next months and to start to generate adenoviral particles that will be used in mice to accomplish the task originally proposed.

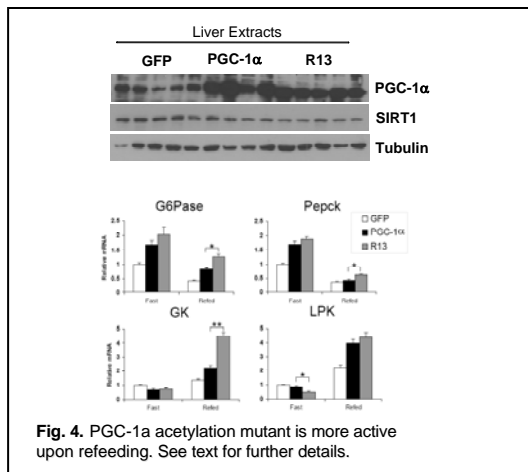
• *Analysis of GCN5 effects on hepatic gluconeogenic/glycolytic, fatty acid oxidation and mitochondrial gene expression depending on PGC-1 α in the liver of obese/diabetic mice. Use of mice injected with adenoviruses encoding GCN5 siRNA and PGC-1 α . (Months 30-48).*

See previous sub-task 5. In this case we have successfully used a PGC-1 α shRNA that was provided by Marc Montminy (Salk Institute, San Diego) (Rodgers et al. 2007). Thus, as soon as we generate adenoviruses encoding for mouse GCN5 shRNA we will be in a position to accomplish this subtask.

• *Analysis of PGC-1 α acetylation to control hepatic gluconeogenic/glycolytic gene expression, fatty acid oxidation and mitochondrial gene expression. Use of mice injected with adenoviruses encoding PGC-1 α and PGC-1 α acetylation mutants. (Months 30-48).*

In this subtask we have successfully deliver adenoviruses encoding for PGC-1 α or PGC-1 α acetylation mutant (R13) to the liver to test the effects on metabolic gene expression.

Although in the previous report we mentioned that R13 is still acetylated by GCN5, we decided



to use R13 because it did not respond to low glucose levels or Sirt1 activators. We think that the additional acetylation sites in R13 might not be physiologically relevant and the fact that mutation of these sites allows acetylation of new sites. However, although not proposed in this project we are pursuing whether additional sites might be regulatory relevant. As shown in Fig. 4 expression of PGC-1 α wild type or mutant R13 were about 3-4 fold compared to GFP infected livers. After 5 days of infection we sacrificed mice and hepatic gene expression was analyzed. Two gluconeogenic genes were analyzed, G-6-Pase and PEPCK in the fasted and refed state. As expected

PGC-1 α wild type induced expression of both genes in these conditions. However, R13 had similar effects as PGC-1 α wild type in the fast state, however upon refeeding R13 expression resulted in higher expression of gluconeogenic genes. Interestingly, PGC-1 α wild type and R13 decreased expression of glycolytic genes in the fasted states, but increased expression in the refed state. Again, in refed conditions R13 increased GK and LPK to higher amounts compared to wild type PGC-1 α .

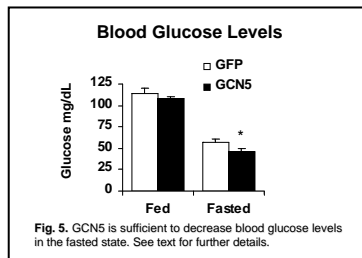
Taken together, these results indicate that PGC-1 α acetylation –that decreases in the fasted state and increases in the refed state- controls expression of gluconeogenic genes, thus in the fasting there is no differences between the two PGC-1 α alleles –this is expected because, PGC-1 α wild type is deacetylated-; however upon refeeding PGC-1 α wild type is acetylated but R13 remains poorly acetylated and is more active increasing expression of those genes. As it relates to the glycolytic genes, it is not clear how PGC-1 α acetylation might directly control these genes. It is likely that the effects are indirect since no direct effects of PGC-1 α on these promoters have been previously reported. The fact, in the R13 refed state both glycolytic and gluconeogenic genes remained elevated (Fig. 4) might indicate that it created a glucose futile cycle, the metabolic consequences of this is not understood. Since we have other tasks to accomplish, we decided not to pursue the implications of this futile cycle, but it might have important

consequences in conditions where PGC-1 α remains constantly de-acetylated. This might also have some implications for humans under stress or starvation creating a locked catabolic state that might compromise blood glucose levels.

Task 6. Physiological and metabolic analysis of GCN5 requirement and PGC-1 α acetylation to control glucose production in liver of obese/diabetic mice. (Months 30-48).

- *Analysis of GCN5 and PGC-1 α acetylation on blood glucose and insulin levels. Measurement of blood glucose and insulin levels in obese/diabetic mice infected with adenoviruses encoding GCN5 siRNA, PGC-1 α siRNA, PGC-1 α and GCN5. (Months 30-48).*

Due to the technical difficulties in generating GCN5 siRNA –see task 5- we have not started these experiments. However, in order to have some preliminary data in this subtask, we injected GCN5 adenoviruses to determine effects on blood glucose levels. Fig. 5 show that under fed



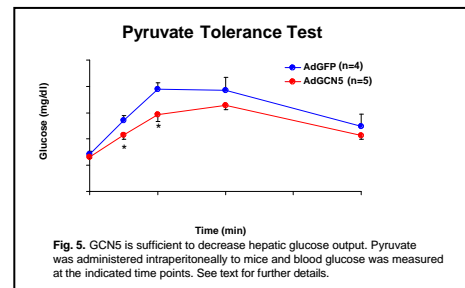
conditions GCN5 expression did not alter blood glucose concentrations. However, under fasting conditions GCN5 was sufficient to decrease blood glucose concentrations, likely through suppression of hepatic glucose output. Again, these results support our model in which PGC-1 α is acetylated under fed conditions but become deacetylated under fasting conditions. Thus, it is expected that GCN5 would not have an effect in fed conditions –since PGC-1 α is already acetylated- but it will have

effects under fasting conditions.

- *Analysis of GCN5 and PGC-1 α acetylation on hepatic glucose production in obese/diabetic mice. Pyruvate tolerance tests in mice infected with adenoviruses encoding GCN5 siRNA, PGC-1 α siRNA, PGC-1 α and GCN5. (Months 30-48).*

Similar to the previous subtask, we have not initiated these experiments due to the lack of GCN5 siRNA adenoviruses availability. However, in the same set of experiments describe above, we determine the ability of hepatic glucose production in mice with hepatic GCN5 overexpression. Using pyruvate tolerance tests, Fig. 6 shows that GCN5 overexpression in liver is sufficient to inhibit hepatic glucose production from pyruvate. These experiments are consistent with the results obtained in the previous subtask and further support our hypothesis that PGC-1 α acetylation is critical to control glucose metabolism.

In the next funded period we will initiate these experiments once we have generated the adenoviruses encoding for GCN5 siRNA. We don't expect major problems since all the reagents and techniques are available in the laboratory and we are in a unique position to accomplish this subtask.



- *Analysis of GCN5 and PGC-1 α acetylation on insulin signaling in obese/diabetic mice. Glucose and insulin tolerance tests in mice infected with adenoviruses encoding GCN5 siRNA, PGC-1 α siRNA, PGC-1 α and GCN5. (Months 30-48).*

As mentioned above in this task 6, we have had technical difficulties to generate a mouse GCN5 siRNA and we have not initiated this sub-task. Our strategic plan is that once this reagent will be generated, -planned in the next months- we will initiate the experiments to accomplish the task as was originally proposed.

Key Research Accomplishments

In this third year, we have accomplished experiments included in tasks 2, 4, 5 and 6 that were originally proposed in the application.

Task 2.- • We have now almost complete task 2, except for additional experiments in the chromatin immunoprecipitation experiments to determine the effects of Wdr18 on PGC-1 α recruitment to promoters. We have demonstrated that Wdr13 and Pc3 are in the PGC-1 α complex through immunoprecipitation analysis. Wdr13 is more specifically in the sub-GCN5 complex and can increase the acetyltransferase activity of GCN5. In addition, the presence or absence of Wdr18 does not affect the integrity of the GCN5 complex subunits. Finally, we show that the interaction of GCN5 and WDR18 is dependent on insulin and Wdr18 is directly phosphorylated by insulin.

Task 4.- • We have demonstrated that Wdr18 is required for full activity of PGC-1 α on gluconeogenic genes, but did not have any effect on glycolytic genes. We have generated Pc3 reagents and adenoviral particles (Pc shRNA) and PGC-1 α acetylation mutant (R13) to use in the next funded period.

Task 5.- • We have started to generate an adenovirus encoding for a mouse GCN5 shRNA. We have determined the effects of the PGC-1 α acetylation mutant on the expression of gluconeogenic and glycolytic genes. We have expressed PGC-1 α wild type and R13 mutant in mice livers and show that R13 is a hyperactive allele of PGC-1 α inducing gluconeogenic genes in the fed state. Interestingly, R13 strongly activates glycolytic genes creating a glucose futile cycle.

Task 6.- • We have shown that hepatic expression of GCN5 is sufficient to decrease blood glucose levels in the fasted state. Moreover, GCN5 is also sufficient to decrease hepatic glucose production in life mice.

Reportable Outcomes

Task 2, 4, 5 and 6. The results that are described in detail in this Research Technical Report have been partially published or are related to the publications indicated below.

A. Coste , J.F. Louet , M. Lagouge , C. Lerin , M. Antal , H. Meziane , K. Schoonjans , **P. Puigserver**, B. O'Malley and J. Auwerx. The genetic ablation of SRC-3 protects against obesity and improves insulin sensitivity by reducing the acetylation of PGC-1 α . *Proc. Natl. Acad. Sci. USA*. 2008 105(44):17187-92.

G. Suchankova, L. Nelson, Z. Gerhart-Hines, M. Kelly, M.S. Gauthier, A.K Saha, Y. Ido, **P. Puigserver** and N.B. Ruderman. Concurrent regulation of AMP-activated protein kinase and SIRT1 in mammalian cells. *Biochem. Biophys. Res. Commun.* 2009 378:836-41.

C. Canto, Z. Gerhart-Hines, J.N. Feige, M. Lagouge, J.C. Milne, L. Noriega, P.J. Elliott, **P. Puigserver** and J. Auwerx. AMPK regulates energy expenditure by modulating NAD⁺ metabolism and SIRT1 activity. *Nature*. 2009. In press.

T.J. Kelly, C. Lerin, W. Haas, S.P. Gygi, and **P. Puigserver**. GCN5-Mediated Transcriptional Control of the Metabolic Coactivator PGC-1 β through Lysine Acetylation. Submitted, 2009.

Additionally, these results have been partially presented in the following conferences or seminars as invited speaker in 2008.

- Keystone Symposia. Diabetes Mellitus, Insulin Action and Resistance. Breckenridge, Colorado.
- Keystone Symposia. Metabolic Pathways of Longevity. Copper Mountain, Colorado.
- Keystone Symposia. Nuclear Receptors: Orphan Brothers, Whistler, Canada.
- American Society of Biochemistry and Molecular Biology. San Diego.
- Molecular Mechanisms Modulating Skeletal Mass and Function. The Banbury Center, Cold Spring Harbor, New York.
- 8th Symposium Molecular and Physiological Aspects of Type 2 Diabetes and Obesity. Nobel Forum, Wallenbergsalen. Karolinska Institutet and Stockholm University. Stockholm, Sweden.
- Baylor College of Medicine. Department of Molecular and Cellular Biology. Houston, TX.
- Case Western Reserve University. Department of Genetics, Cleveland, OH.
- University of Michigan Medical School. Department of Molecular and Integrative Physiology. Ann Harbor, MI.
- University of California, Los Angeles. Department of Molecular Medicine and Pharmacology. Los Angeles, CA.
- University of Southern California. Department of Pharmacology and Pharmaceutical Sciences. Los Angeles, CA.
- Colloquium on the Biology of Aging. Marine Biological Laboratory, Woods Hole, MA.
- Wyeth Symposium on Metabolic Dysregulation. Boston University, Department of Pharmacology and Experimental Therapeutics. Boston, MA.
- The Hamner Institutes for Health Sciences. Research Triangle Park, NC.
- University of Geneva. Faculty of Medicine. Department of Cell Physiology and Metabolism. Geneva, Switzerland.

Conclusion

We have summarized in this Research Technical Report the experiments and results that have been obtained in the third year of this Award. We have accomplished the original tasks that were proposed and we will continue in the next year to accomplish the planned tasks. We have generated additional siRNAs for Pc3 and WDR-18 (we have at this moment adenoviruses for both gain- and loss-of-function), and we are in the process of generating mouse GCN5 siRNA that will allow us to accomplish the next tasks that were proposed in SOW. We have demonstrated that Pc3 and Wdr18 are part of the PGC-1 α complex. Wdr18 is part of the GCN5

complex and directly control the enzymatic activity of GCN5 that leads to PGC-1 α acetylation and inactivation. In cultured hepatocytes, Wdr18 is required for the maximum transcriptional activity and expression of gluconeogenic genes, indicating that Wdr18 acts as a suppressor of PGC-1 α on those genes. However, the expression of hepatic glycolytic genes is not affected by expression of Wdr18. In vivo GCN5 is sufficient to decrease blood glucose levels and suppress, at least in part, hepatic glucose output. Consistent with these effects, expression of a PGC-1 α acetylated mutant (not sensitive to nutrient-dependent acetylation changes) is hyperactive increasing genes of gluconeogenesis and glycolytic genes. Taken together, the results presented in this Research Technical support are consistent with our overall hypothesis that PGC-1 α acetylation is a key chemical modification that control glucose and lipid metabolism. In the next year we will continue our studies to show the functionality of this pathway in different metabolic conditions in vivo. Our long-term aim is to provide the basis for therapeutical targets that can be use to prevent glucose and lipid dysregulation that occur in conditions of stress or overnutrition that have a significant impact in the personnel army.

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Appendices

None.