A Novel Role of the Anticoagulant Protein S in Preventing Thrombosis

Logan Barrios[1], Verima Pereira, PhD[2], Rinku Majumder, PhD[2].

[1]University of Notre Dame Department of Biological Sciences, [2]Louisiana State University Health Sciences Center Department of Biochemistry and Molecular Biology.

Introduction

Obesity is a major health threat to the global population. About 13% of the world's population is obese, and more than 42% of the United States population is obese [1]. In obesity-induced diseases, the liver becomes hypoxic. Hypoxia stabilizes transcription factor Hypoxia Inducible Factor 1α (HIF1α) by preventing its degradation [2]. Recently, work from Dr. Majumder's lab showed that an increase in HIF1α expression downregulates Protein S (PS) expression in HepG2 cells (Figure 2) [3]. PS is a vitamin K-dependent anticoagulant that is synthesized in the liver. PS has three known functions in coagulation, the most important being that it directly inhibits factor IXa in the blood coagulation pathway [4]. This is important because PS deficiency increases the risk of venous thrombosis. This effect explains why obesity increases the risk of thrombosis. **The goal of this project was to determine whether hypoxia associated with obesity results in a prothrombotic state because of the downregulation of PS.** Additionally, it has been found that the risk of thrombosis increases as much as 24-fold in obese individuals who use oral contraceptive agents (OCAs) [5]. Estrogen decreases plasma PS levels as much as 2-3 fold. Estrogen suppresses PS levels by inhibiting PS gene transcription; estrogen receptor α and transcription factor SP1 mediate this transcriptional inhibition [6]. Therefore, acquired PS deficiency occurs in women who use estrogen-based OCAs are at a greater risk for thrombosis. As a result of this increase in thrombotic risk, **we also determined OCAs and obesity synergize to reduce PS levels in human plasma**.

Methods

In this project, we used three mediums to test our hypothesis: HepG2 cells, mice plasma, and human plasma.

HepG2 cells were grown at different concentrations of O_2 for 4 hours in order to replicate hypoxic conditions. We assessed the PS levels by immunoblotting each of the HepG2 cells at the different concentrations of O_2 (Fig. 2A). We also analyze the effect of hypoxia on regulating PS expression in vitro (Fig. 2B & C).

Next, we monitored mice (n=40) and their weight gain over 4 months (Fig. 3). Control mice were on a lean diet (n=20), and obese mice were given a high fat diet (n=20). Upon completion of inducing obesity in the mice, we extracted mice plasma to perform thrombin generation assays (Fig. 1; Fig. 4) and ELISA assays (Fig. 5). Additionally, we used plasma from control mice, HIF1α knockout mice, and the HIF1α P564A mutant (HIF1α dPA) mice, which overexpresses HIF1α and stimulates more hypoxic conditions than in normal O_2 conditions. This allowed us to show the effect of HIF1α on PS and thrombin generation. Plasma was extracted from each of the three types of mice and immunoblotted to measure the liver and plasma PS levels (Fig. 6A & B). We measured thrombin formed by these plasma samples with a thrombin generation assay (Fig. 6C).

Finally, we obtained plasma from normal, obese, and obese individuals who use oral contraceptives. We used ELISA assays to measure the total and free PS levels in each category of individuals (Fig. 7A & B). CYROcheck Clot S™ from Precision Biologic (CCS-30) allowed us to analyze the free PS activity. We measured the peak thrombin of each category of individuals by thrombin generation assay (Fig. 7C). We compared thrombin generation and PS levels with the clotting time of each group (Fig. 7D).

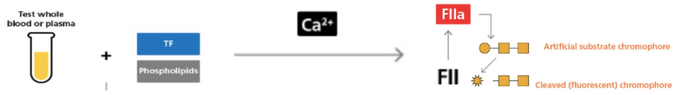

Figure 1: Schematic of the thrombin formation reaction and cleavage of the fluorescent substrate in thrombin generation assay.

HepG2 Cell Results

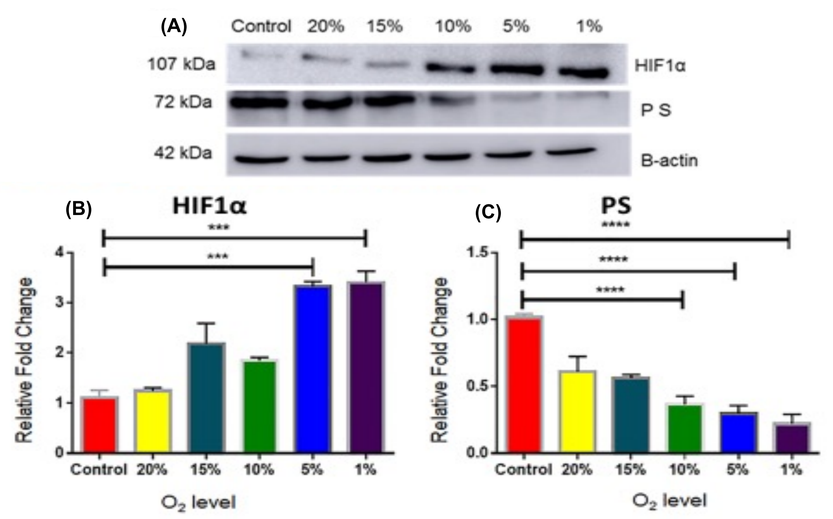

Figure 2: Hypoxia regulates PS expression in vitro. HepG2 cells were grown at different concentrations of O_2 for 4 hours. A) Representative immunoblots showing relative PS and HIF1α protein levels. B) Relative HIF1α mRNA levels. C) Relative PS mRNA levels. ****P<0.0001.

Mice Results

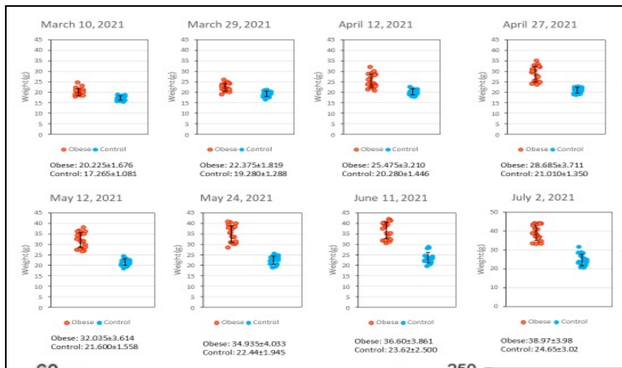

Figure 3: Mice weight over the course of 4 months. 20 control mice received a lean diet. 20 mice received a high fat diet to induce obesity. After obesity was induced and the weight was consistent, plasma from both groups of mice was extracted.

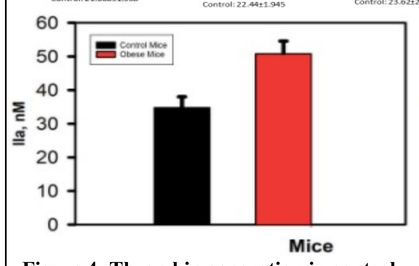

Figure 4: Thrombin generation in control and obese mice. Peak thrombin was greater in obese mice plasma than control.

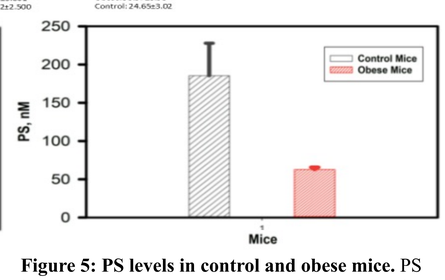

Figure 5: PS levels in control and obese mice. PS levels measured by ELISA assays shows PS levels are downregulated in obese mice plasma compared to control.

dPA and Knockout Mice Results

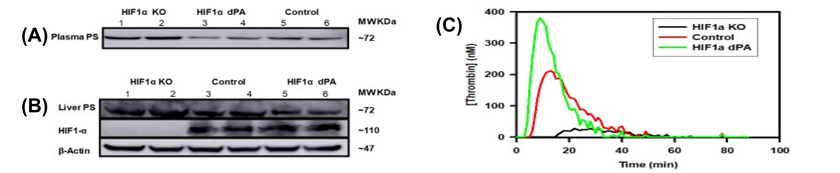

Figure 6: Effect of HIF1α on PS expression and thrombin generation in HIF1α liver-specific KO and dPA mice. From HIF1α liver-specific KO and dPA mice and normal mice A) Citrated plasmas were analyzed by immunoblotting for relative PS levels. B) Liver extract immunoblot analysis for relative PS levels and HIF1α expression (Panel 1. Liver PS, Panel 2. HIF1α, and Panel 3. β –Actin). C) Citrated plasmas were assayed for thrombin generation.

Human Results

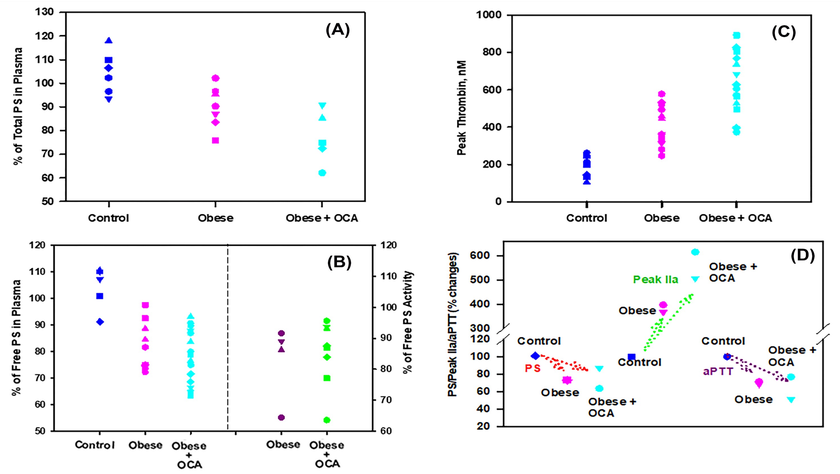

Figure 7: Obesity and estrogen decrease plasma PS level and activated partial thromboplastin time (aPTT), thereby elevating thrombin generation. Plasma was collected from normal, obese, and obese subjects who use oral contraceptive agents. A) total and B) free PS levels/PS activity. Total and free PS levels were measured by ELISA (Diapharma 036-001 & 051-001 respectively) and free PS activities were measured by CYROcheck Clot S™ from Precision Biologic (CCS-30). C) Peak thrombin generation measured as described in Figure 1. Obese subjects and obese subjects who used OCA showed higher peak thrombin generation indicating a higher risk of thrombosis. D) Overall percent changes in free PS and peak thrombin generated were demonstrated for obese subjects and obese subjects with OCA compared with normal subjects.

Conclusions

We observed that hypoxia associated with obesity downregulates PS and increases the thrombotic risk. In obese mice, we observed upregulation of thrombin generation and PS levels were downregulated. Additionally, the HIF1α dPA mice produced more thrombin than the control mice and the HIF1α KO mice. We also demonstrated the combination of obesity and OCAs increases thrombotic risk through the downregulation of PS shown in the human plasma data.

Completion of these studies will provide understanding of the regulation of PS expression. Using the mice studies allows us to closely model the human physiological process, which will help us when testing therapeutics for PS supplementation. This project will be instrumental in investigating new antithrombotic strategies for chronic complications that have high thrombotic risk, such as obesity and for those who require the use of OCAs.

This research project was supported by Award Number: DBI-2051440 through the National Science Foundation (NSF), Research Experiences for Undergraduates (REU) Program and by grant 1R01HL151613-01A1 through the National Institutes of Health.

References:

1. "Obesity and overweight." World Health Organization, 2021. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight
2. Semenza, GL, "Hypoxia-inducible factor 1 (HIF-1) pathway." Sci STKE, 2007. (407):cm8.
3. Pilli VS, Datta A, Sadaf A, Catalano D, Szabo G., Majumder, R., "Hypoxia downregulates protein S expression." Blood 2018. 132, 452-455, 132, 348-349 PubMed PMID: 29784640
4. Chattopadhyay, R., Sengupta, T., and Majumder, R., "Inhibition of Intrinsic Xase by Protein S - a novel regulatory role of Protein S independent of Activated Protein C." Arteriosclerosis, Thrombosis, and Vascular Biology, 2012. 32(10) : 2387-2393.
5. Pomp, E.R., et al., "Risk of venous thrombosis: obesity and its joint effect with oral contraceptive use and prothrombotic mutations." Br J Haematol, 2007. 139 (2): p. 289-96.
6. Suzuki, A., et al., "Down-regulation of PROS 1 gene expression by 17beta-estradiol via estrogen receptor alpha (ERalpha)-Sp1 interaction recruiting receptor-interacting protein 140 and the corepressor-HDAC3 complex." J Biol Chem, 2010. 285(18): p. 13444-53.