"Nitric Oxide and TNF-alpha Production by the Pro-Inflammatory iNOS Pathway in Activated Macrophages from Rats with Inactivated Melanocortin Receptor Genes"

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Abstract

Obesity is characterized by many different components, one of which is chronic, low grade inflammation. This inflammation is caused by the elevated levels of macrophages in the adipose (fat). The macrophages create a pro-inflammatory response using the inducible nitric oxide synthase pathway, a cellular process that causes the production of nitric oxide (a powerful vasodilatory compound) and tumor necrosis factor-alpha (a pro-inflammatory cytokine). A protein called alpha-melanocyte stimulating hormone acts on melanocortin receptors on the macrophages to downregulate this pro-inflammatory immune response. Therefore, I hypothesized that white adipose tissue from rats with fully inactive genes for melanocortin receptors would have significantly higher levels of nitric oxide and tumor necrosis factor-alpha, and that similar tissue samples from rats with partially knocked out genes would have slightly lower levels of nitric oxide and tumor necrosis factor-alpha but that these would be still higher levels than tissue samples from wild type rats (normal). To test this hypothesis, I used tissue samples from rats with fully knocked out melanocortin receptor genes, partially inactivated melanocortin genes, and wild type genes. These were quantitatively analyzed for variations in nitric oxide levels using a colorimetric Griess reagent test, and for variations in TNF-alpha levels using a Bradford assay for normalizations and an enzyme-linked immunosorbent assay (ELISA).

While the results were not what I expected, they still yielded potentially useful information. The Griess reagent test demonstrated no detectable levels of nitric oxide and the ELISA showed that there were no statistically significant differences in tumor necrosis factor-alpha levels in the three groups of rats.

Introduction

Obesity levels around the world and in this country (see Appendix I) have skyrocketed in recent years and scientists are just beginning to realize how little we know about its causes and its effects on the human body. While much research has been performed and continues to be performed today, the complex relationship between the excess adipose tissue and the body's organ systems and signaling molecules remains only partially understood.

Obesity is a chronic disease defined by the excessive growth of adipose tissue, in both quantity and its ability to store triglycerides, thus producing a wide variety of effects characterized under the general heading of "metabolic syndrome" (Leal 87; Antuna-Puente 2; Larsen). Metabolic syndrome is characterized by the presence of three or more of the following: high blood pressure, high blood sugar, low levels of HDL, high triglyceride levels, or the presence of above normal amounts of abdominal fat (Lim 29). These may lead to further complications such as Type II diabetes, cardiovascular diseases, pulmonary diseases, musculoskeletal diseases, genitourinary diseases in women, neurologic diseases, cataracts, and gastrointestinal diseases (Larsen).

Adipose tissue (see Appendix 2) was originally thought to have no other function than to store triglycerides for energy. Now, however, it is viewed as a complex and potentially highly active endocrine organ. Not only are the adipocytes themselves capable of producing cytokines (messenger proteins that participate in cellular communication) and other various types of signaling proteins, but the activated macrophages that infiltrate the tissue do so as well and together they propitiate a chronic state of low grade inflammation (Larsen).

Part of this inflammatory response is caused by the presence of elevated numbers of macrophages in adipose tissue, whose presence is necessitated by their apparent function of scavenging moribund adipocytes (Greenberg 461S). These macrophages produce a number of cytokines—such as interleukins-1, -6, interferon-gamma, tumor necrosis factor-alpha (TNF-alpha) and others—all of which induce a pro-inflammatory response. The surrounding macrophages respond to the increased TNF-alpha levels by producing nitric oxide, a powerful vasodilator that serves to propitiate the inflammatory response and decreases blood pressure (Rajora). The counterbalancing anti-inflammatory cytokines (transforming growth factor-beta, and interleukins- 4, -10, -13, -35) that the macrophages also may produce fail to completely down-regulate the inflammatory response produced by the pro-inflammatory cytokines (see Appendix 3 for sources and functions of cytokines) (Tortora). The result of this is that the homeostatic balance between pro-inflammatory and anti-inflammatory proteins is upset and many tissues in obese individuals exhibit symptoms that are characteristic of inflammation, such as heat, swelling, and vasodilation.

The production of the pro-inflammatory cytokines and other substances by either the macrophages present in the adipose tissue or the adipocytes themselves result not only in inflammation, but ultimately plays a role in in the development of insulin resistance, type II diabetes, and other components of metabolic syndrome, such as an increased risk of cardiovascular disease (Antuna-Puente 2).

Nitric oxide is produced by the inducible nitric oxide synthase (iNOS) pathway in the macrophages present in the adipose tissue, thus continuing the inflammatory response and stimulating the release of additional TNF-alpha. This pro-inflammatory

pathway is named as such because it utilizes iNOS as a catalytic enzyme for the oxidation of cellular compounds into nitric oxide (see Appendix 4) (Cianchi 793).

A tridecapeptide protein called alpha-melanocyte stimulating hormone (alpha-MSH) is also present in the adipose tissue and acts to regulate the pro-inflammatory iNOS pathway by inhibiting the expression of the enzyme iNOS and ultimately the production of nitric oxide and additional TNF- alpha while modulating several other immune responses (Star 8016). This hormone performs a number of functions in the body (as does its receptors), but is well documented as a potent immunomodulator. The alpha-MSH functions by binding to MCR-1 melanocortin receptors on monocytes, macrophages, and dendritic cells and, in the case of macrophages, it causes the cell to inhibit the expression of iNOS (Luger 133).

Without the necessary melanocortin receptors, however, alpha MSH is unable to properly downregulate the pro-inflammatory response of the adipose tissue; therefore, the iNOS pathway may be only partially suppressed, or completely uninhibited, if some or all of the melanocortin receptors are not expressed (see Appendix 4). I hypothesized that white adipose tissue from rats with fully knocked out genes for melanocortin receptors will have significantly higher levels of nitric oxide and TNF-alpha, whereas similar tissue samples from rats with partially knocked out genes will have slightly lower levels of nitric oxide and TNF-alpha but still have higher levels than tissue samples from wild type rats.

To test this hypothesis, white adipose tissue samples were taken from rats with fully knocked out melanocortin receptor genes, partially knocked out melanocortin genes, and wild type genes. These were quantitatively analyzed for variations in nitric oxide

levels using a colorimetric Griess reagent test, and for variations in TNF-alpha levels using a Bradford assay for sample dilution standardization and an ELISA assay for identification and quantification of TNF-alpha levels. The role of pro-inflammatory signaling molecules and chemical compounds is an important one—particularly in relation to how the resulting inflammation affects the body and contributes to further medical complications of obesity—but it is still not fully understood and requires continued research efforts.

Methods

Sample Conditions

The tissue samples used in this research were from Dr. Colleen Novak's rat lab at Kent State (Dept. of Biology). The rats were sacrificed through decapitation and the tissues were collected, wrapped in foil, and flash frozen before being placed in a freezer for long-term storage at which time, they were thawed. The source of the white adipose tissue used in this research was the epididymis of male rats of an inbred line of laboratory rats.

The Griess Reagent Test

Around 0.5 grams of adipose tissue were taken from samples from a wild type rat, a rat with partially knocked out melanocortin receptor genes, and a rat with fully knocked out melanocortin receptor genes. The tissues were placed in a Dounce homogenizer and enough deionized water was added to cover the tissue. The tissue cells were lysed and the sample was homogenized by approximately 20 repetitions of the homogenizer. Using a pipette, 1 mL of the homogenized tissue sample was placed in a 2 mL Eppendorf tube and 1 mL of 1x Griess reagent was added. The tube was inverted several times and allowed to sit for 15 minutes at room temperature before results were noted.

Because nitric oxide in cells is unstable and tends to rapidly break down, it can be very difficult to measure nitric oxide levels in tissue, however, the primary product of the decomposition reaction is nitrite, a stable end product that is easily measurable using the Griess reagent (see Appendix 6).

The Bradford Protein Assay

Approximately 0.5 grams adipose tissue samples were taken from 18 rats for use in the Bradford and ELISA assays. Six of the rats were wild type, six had partially knocked out melanocortin receptor genes, and six had fully knocked out melanocortin receptor genes. Each sample was lysed in 1 mL of HNTG Lysis Buffer with 20 repetitions in a Dounce Homogenizer. The samples were transferred to 1.5 mL Eppendorf tubes and centrifuged at 4° C 13,000 rpm. A cake of fat was formed at the top of the tubes and was removed using a micropipette. 5 microliters of the supernatant from each tube were pipetted into a 96-well plate and protein standards were created using serial dilutions of bovine serum albumin with both positive and negative controls. The Bradford reagent (250 microliters) was then added to each well being used. The plate was put on a shaker to be mixed for approximately 30 seconds. The samples were then incubated at room temperature for approximately 10 minutes. The absorbance was then measured in a SPECTROstar Nano plate reader at 595 nm. The absorbances were then used to determine the total protein concentration of the standards and a standard curve was created to determine the total protein content of each of the 18 samples.

The TNF-alpha ELISA

Using the individual sample protein contents from the Bradford Assay, an appropriate dilution was calculated for each sample in order to make the total protein concentrations equal. A 96 well plate TNF-alpha ELISA was purchased from Abcam, including all necessary reagents. The protein standards were created using the reagents included with the kit. One hundred microliters of each standard were pipetted into the

first column of wells, along with a positive and negative control. In order to achieve more accurate results, the samples were run in duplicate and 100 microliters of each sample was put into consecutive wells. The plate was covered with Parafilm and incubated at room temperature on a shaker overnight. The plate was then emptied and washed four times with an ELISA specific wash solution. In order to achieve the most accurate results the plate was inverted and blotted after each wash. One hundred microliters of Biotinylated TNF-alpha Detection Antibody was added to each well and the plate was incubated at room temperature for 1 hour with gentle shaking. The plate was then inverted and blotted before being washed four more times with the wash solution. One hundred microliters of HRP-Streptavidin solution was added to each well and the plate was incubated for 45 minutes at room temperature with gentle shaking. The plate was then inverted and blotted before being washed four more times with the wash solution. One hundred microliters of TMB One-Step Substrate reagent were added to each well and the plate was incubated for 30 minutes at room temperature in the dark with gentle shaking. Fifty microliters of Stop Solution were then added to each well and the absorbance was then immediately measured in a SPECTROstar Nano plate reader at 450 nm. The absorbances were then used to determine the TNF-alpha concentration of the standards and a standard curve was created to determine the TNF-alpha content of each of the 36 wells.

Results and Analysis

The Griess Reagent Test

The Griess reagent test is a colormetric assay for nitric oxide, meaning that the presence of nitric oxide will cause a color change of the sample. Though the Griess reagent was confirmed to be viable using a positive control, the samples that were tested showed no color change. This indicates that there was not a measurable amount of nitric oxide present in the tissue, provided that all protocols for the Griess reagent test were performed accurately.

The Bradford Protein Assay

Because the amount of tissue used in each sample cannot be measured to the needed degree of accuracy, the concentration of all proteins in the samples must standardized. This allows the ELISA assay to accurately determine how much of the total protein is TNF-alpha.

The purpose of the Bradford assay is standardization of protein concentration for the ELISA. Based on the calculated concentrations of created standards, the total protein concentrations of the samples were extrapolated and the appropriate dilutions were calculated.
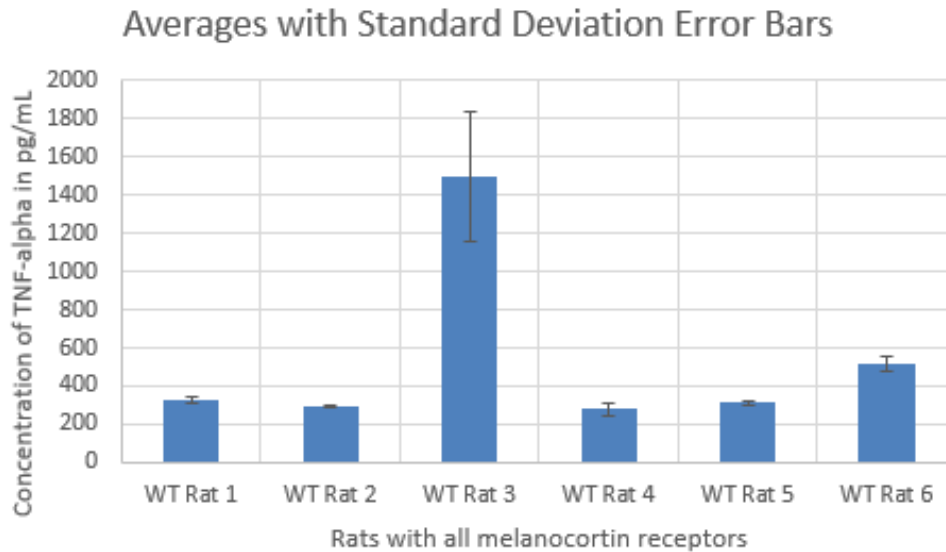
The TNF-alpha ELISA

Figure 1. The averages of the TNF-alpha levels (pg/mL) in the samples from each rat
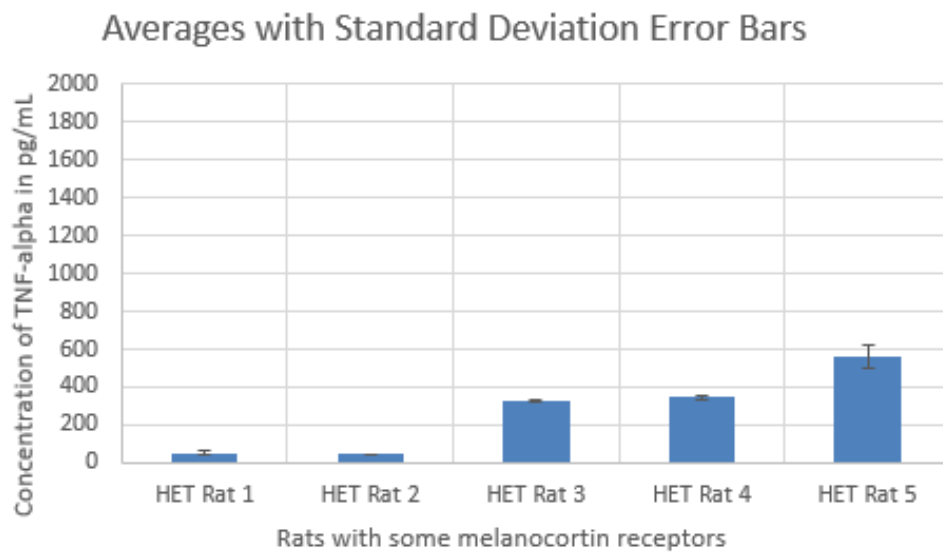
with wild type genes is shown above.

Figure 2. The averages of the TNF-alpha levels (pg/mL) from each rat with heterozygous

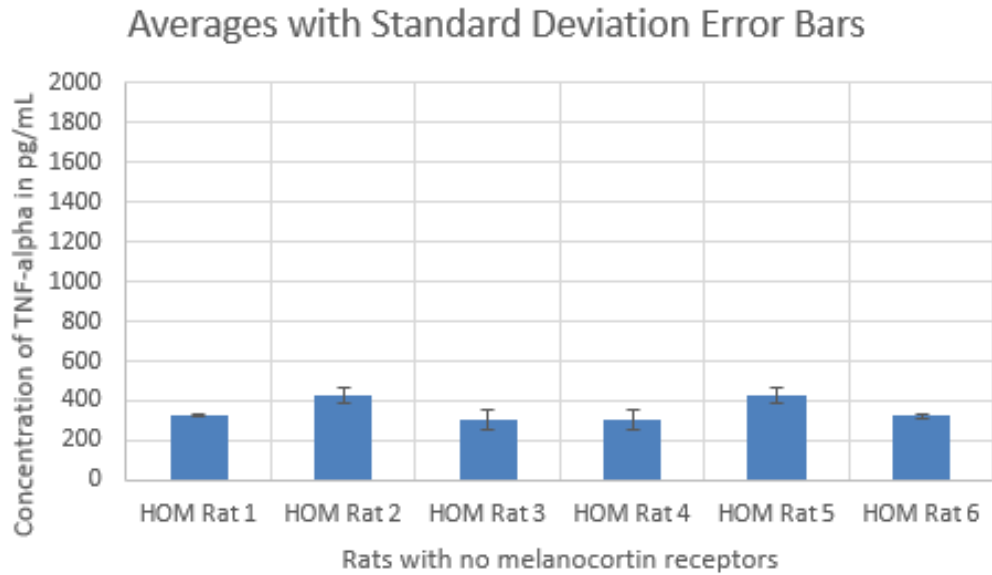melanocortin receptor genes (partially knocked out genes) is shown above.

Averages with Standard Deviation Error Bars

Figure 3. The averages of the TNF-alpha levels from each rat with homozygous

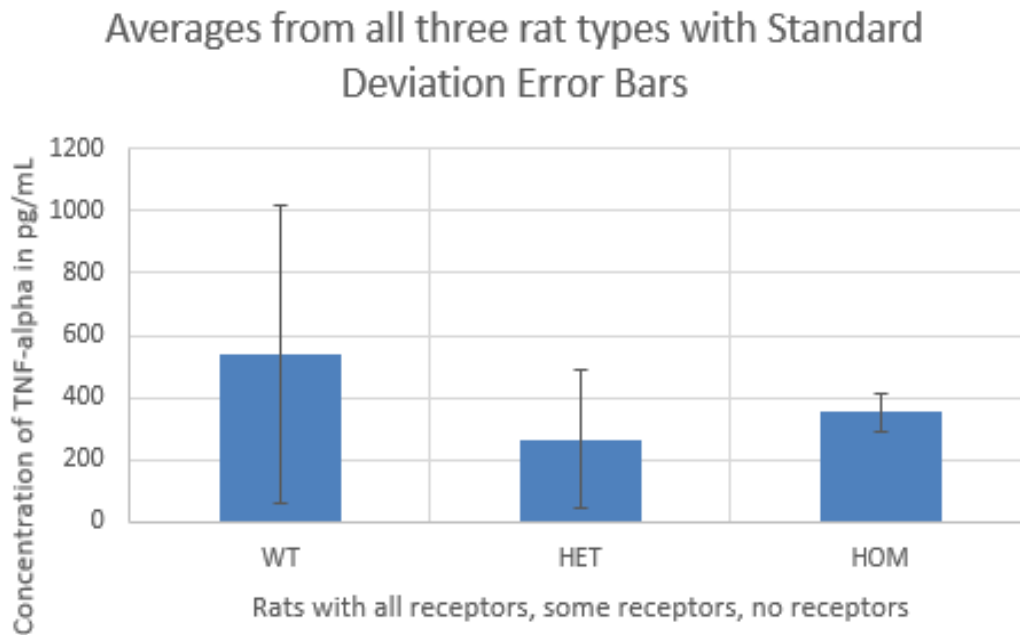melanocortin receptor genes (fully knocked out genes) is shown above.

Averages from all three rat types with Standard Deviation Error Bars

Figure 4. The averages of the TNF-alpha levels from the sample groups is shown above.

See Appendix 7 for the steps of an ELISA

Discussion

While these experiments did not yield the expected results, the results were nonetheless interesting. I hypothesized that white adipose tissue from rats with fully knocked out melanocortin receptor genes would have significantly higher levels of nitric oxide and TNF-alpha than rats with partially knocked out genes and even higher levels than tissue samples from wild type rats.

Putting the data from the TNF-alpha ELISA through the Student's T test yielded the following results in which a number less than 0.05 indicates statistically significant difference:

> Wild Type and Heterozygous: 0.276
>
> Heterozygous and Homozygous: 0.410
>
> Wild Type and Homozygous: 0.371

Based on the results of the T tests, none of the differences in TNF-alpha levels among the three groups were statistically significant. There is no data from the Griess reagent test because sample absorbances were below the range detectable.

There are several potential reasons for the results. Firstly, the sample size may be too small. A larger sample size may yield results that are statistically significant. Secondly, while prior research indicates that melanocortin receptors have a role in downregulating production of TNF-alpha and nitric oxide, a good deal of this research was done using human macrophages, monocytes, and even microglial cells. It has been proven that unlike murine macrophages, human monocytes produce little nitrite upon activation by interferon-gamma (Rajora 248). This indicates that there are in fact distinct

differences between human and murine cells that may affect cellular inflammatory responses. Finally, the macrophages used in prior reported research seem to be primarily activated using interferons, lipopolysaccharide, or other similar antigens in vitro. Using macrophages that have been activated by adipose tissue may affect TNF-alpha and nitric oxide production in vivo.

Potential topics for further research

There are many other related topics that could be done as a follow up to this project. Not only could other components of obesity be considered, but also variations on the research done here.

One potential project for future research is to repeat the Griess reagent test and the TNF-alpha assays white adipose tissue from high capacity runner (HCR) rats and low capacity runner rats (LCR). Rather than having genotypic differences (such as knock out gene rats), these rats have phenotypic differences. The HCR rats have lean body types and have a high tolerance for exercise while the LCR rats have obese body types and a low tolerance for exercise.

Even repeating the test and assays on different types of tissues could yield significant differences. I would expect tissue samples from the pancreas could give insight to insulin resistance and its relation to inflammation. Brain tissue samples might show a relation to appetite hormones such as leptin. There are many other types of cytokines produced by macrophages and other cell types that could be assayed.
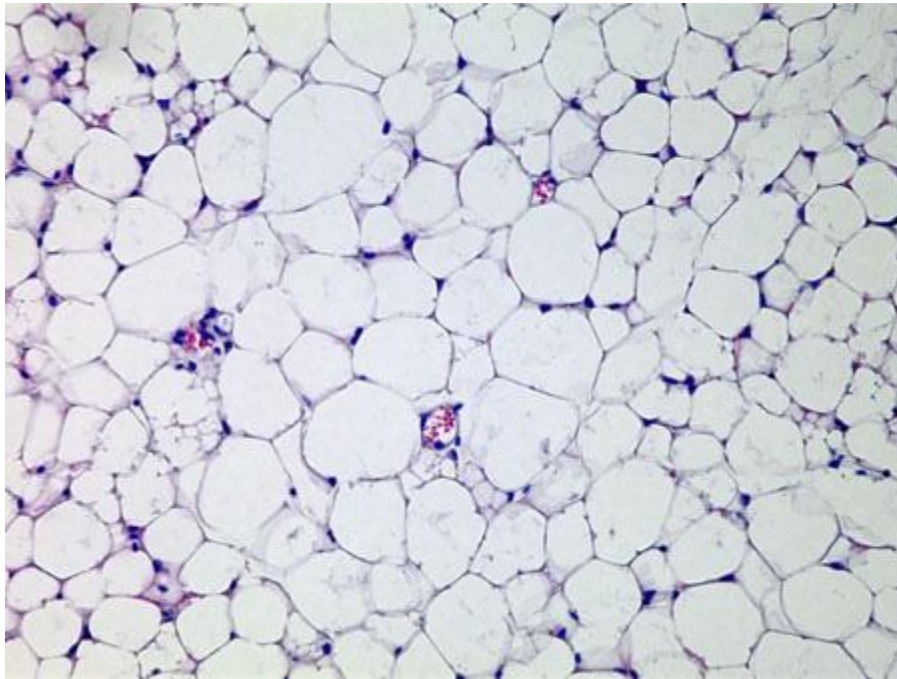
Appendices

Appendix 1: The Prevalence of Obesity

http://www.cdc.gov/obesity/data/prevalence-maps.html
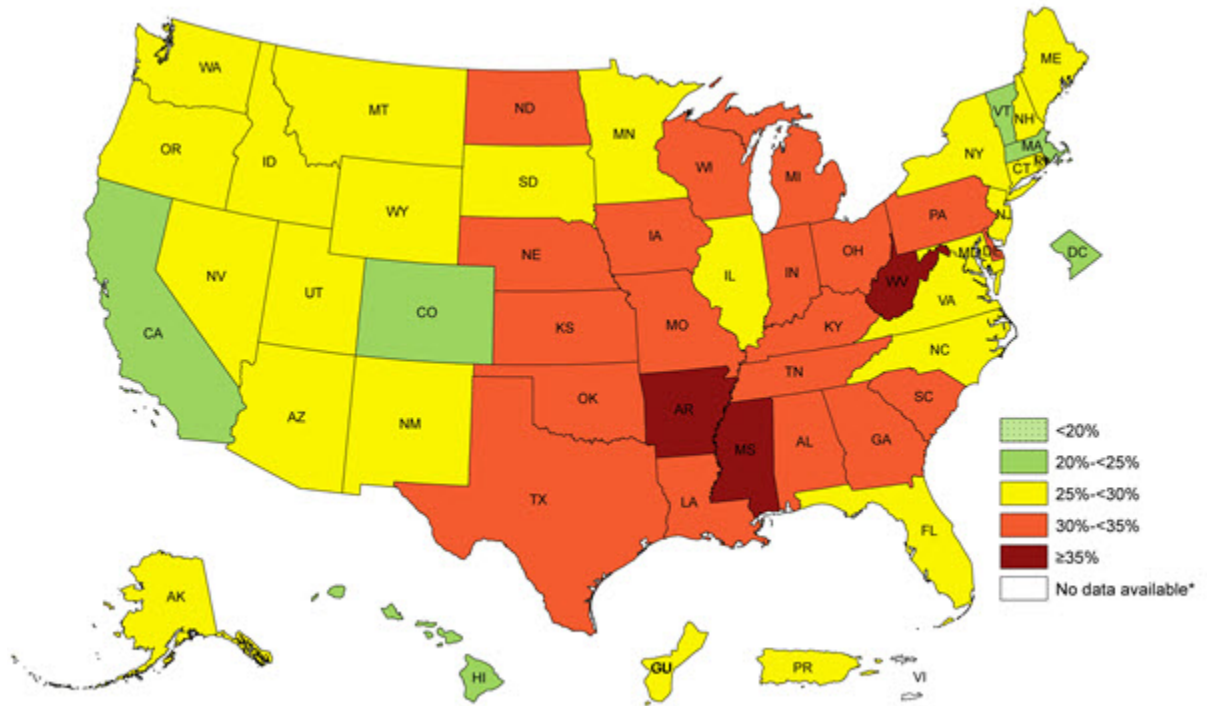
Appendix 2: White Adipose Tissue

www.studyblue.com

Appendix 3: Common Pro-Inflammatory Compounds

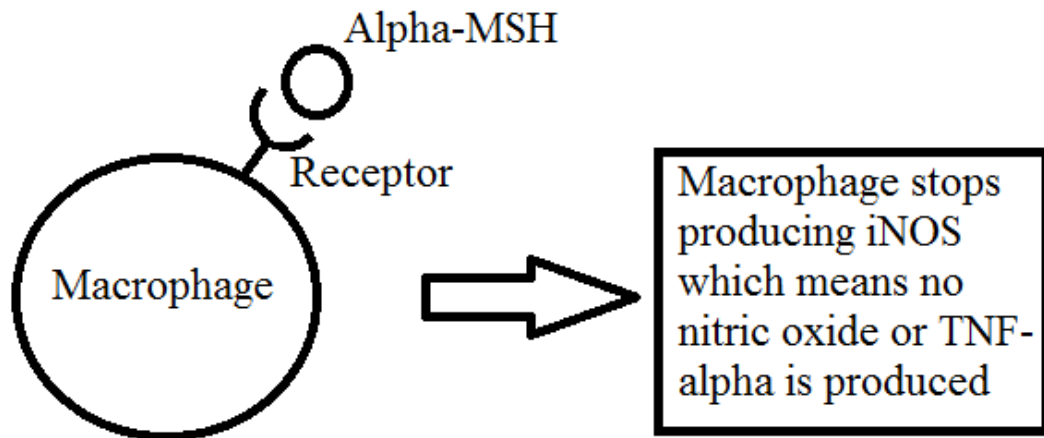| Cytokine	Cell Source	Cell Target	Primary Effects
IL-1	Monocytes Macrophages Fibroblasts Epithelial cells Endothelial cells Astrocytes	T cells; B cells Endothelial cells Hypothalamus Liver	Costimulatory molecule Activation (inflammation) Fever Acute phase reactants
IL-6	T cells; Macrophages; Fibroblasts	T cells; B cells Mature B cells Liver	Costimulatory molecule Growth (in humans) Acute phase reactants
IFN-gamma	T cells; NK cells	Monocytes Endothelial cells Many tissue cells - especially macrophages	Activation Activation Increased class I and II MHC
TNF-alpha	Macrophages; T cells	Similar to IL-1	Similar to IL-1
IL = interleukin GM-CSF = granulocyte-macrophage colony stimulating factor IFN = interferon TNF = tumor necrosis factor TGF = transforming growth factor			

http://www.microbiologybook.org/mobile/m.immuno-13.htm

Appendix 4: An Overview of the iNOS Pathway

http://physrev.physiology.org/content/84/3/731
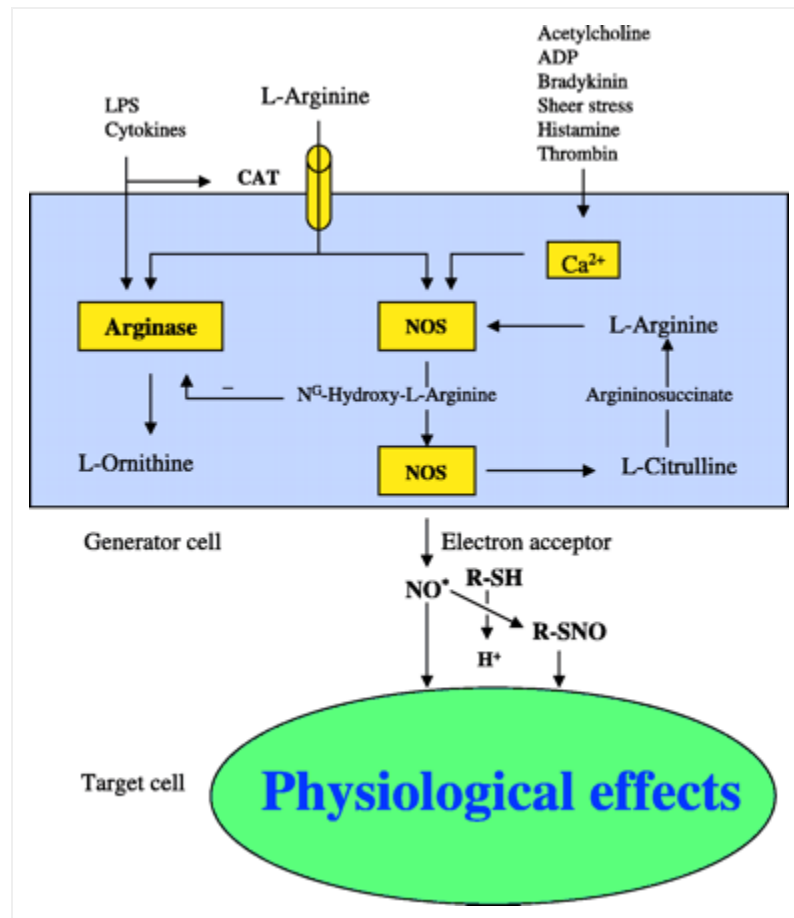
Appendix 5: The Presence and Absence of Melanocortin Receptors

Alpha-MSH

Receptor

Macrophage

Macrophage stops producing iNOS which means no nitric oxide or TNF-alpha is produced

With MCR-1 present

Alpha-MSH

Macrophage

Macrophage continues to make high levels of iNOS which produces more nitric oxide and TNF-alpha
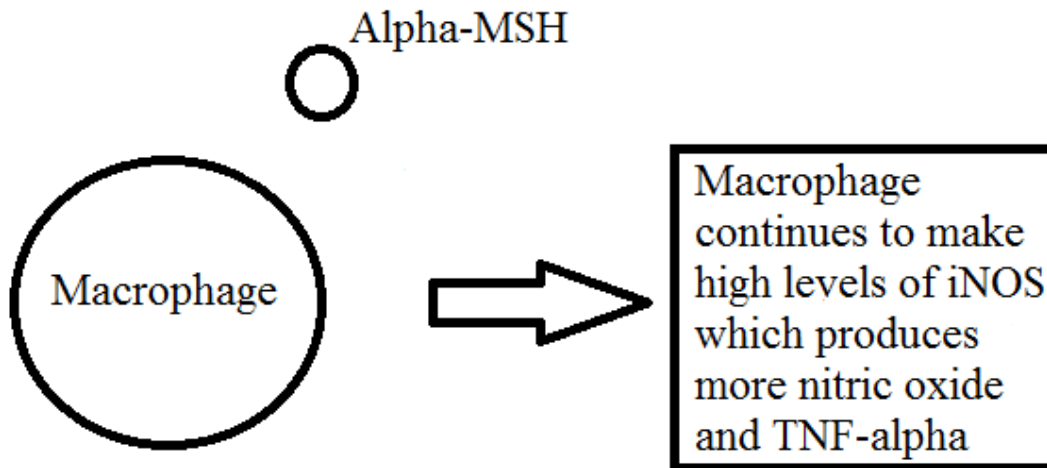
With MCR-1 absent

Appendix 6: Steps of the Griess Reagent Test

Nitrite (NO$_2$)

+ Sulfanilic Acid
(Nitrate Reagent A)

↓

Colorless
(Nitrite-Sulfanilic Acid)

+ alpha-naphthylamine
(Nitrate Reagent B)

↓

Red Precipitate
(Prontosil)

www.cdc.gov
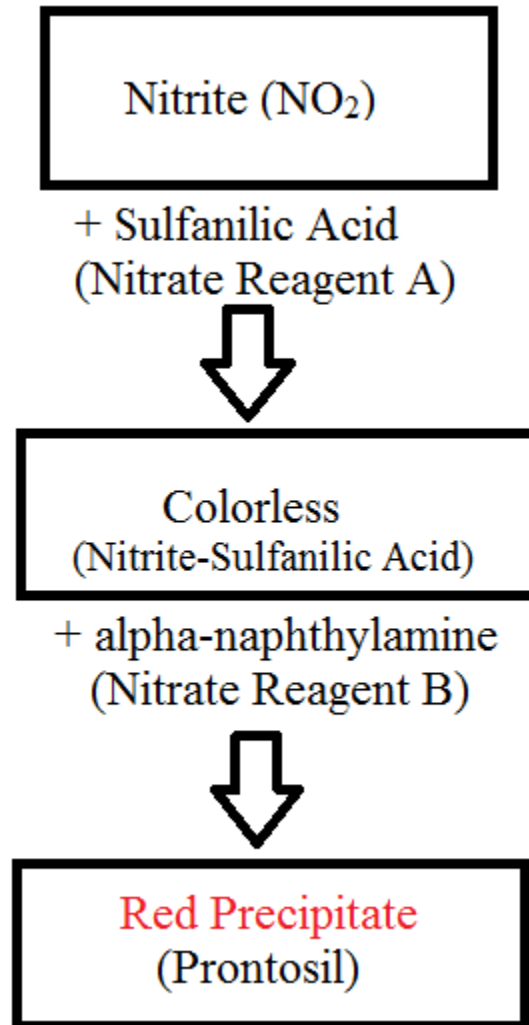
Appendix 7: Steps of an Indirect ELISA

http://www.microbiologynotes.com/elisa-principle-types-and-applications/
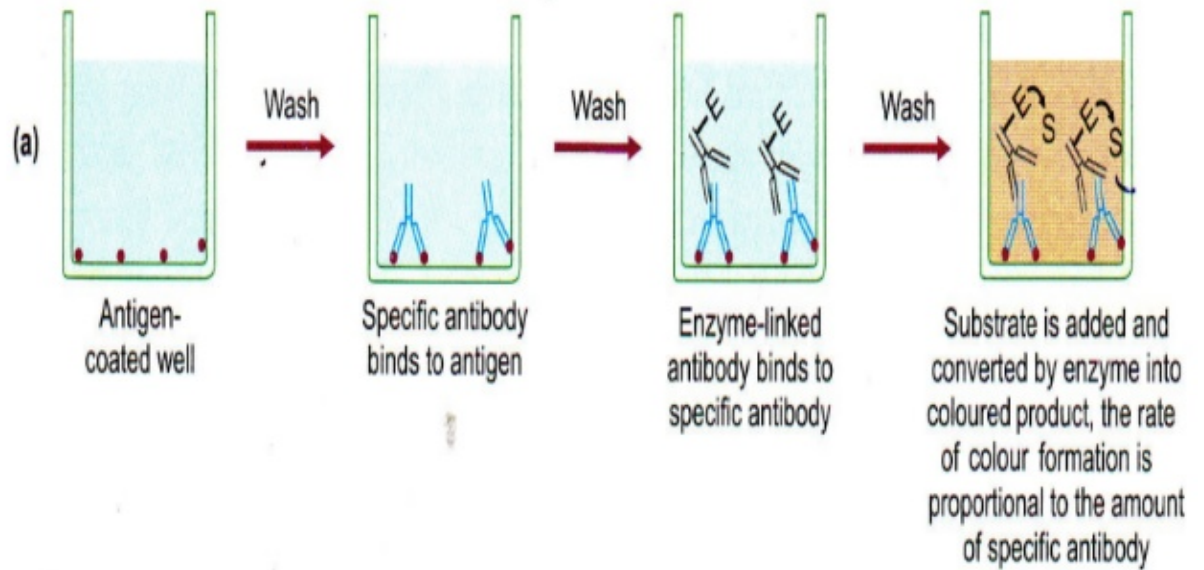
References

Antuna-Puente, B., B. Feve, S. Fellahi, and J.-P. Bastard. "Adipokines: The Missing Link

    between Insulin Resistance and Obesity." *Diabetes & Metabolism* (2007): 2-11.

    Print.

Cianchi, Fabio et al. "Inducible Nitric Oxide Synthase Expression in Human Colorectal

    Cancer Correlation with Tumor Angiogenesis." *The American Journal of*

    Pathology 162.3 (2003): 793–801. Print.

Greenberg, Andrew S., and Martin S. Obin. "Obesity and the Role of Adipose Tissue in

    Inflammation and Metabolism." The American Journal of Clinical Nutrition, Feb.

    2006. Web. 6 Apr. 2016.

Leal, Viviane De Oliveira, and Denise Mafra. "Adipokines in Obesity." *Clinica Chimica*

    Acta (2013): 87-94. Print.

Lim, Soo, and Robert H Eckel. "Pharmacological Treatment And Therapeutic

    Perspectives Of Metabolic Syndrome." *Reviews In Endocrine & Metabolic*

    Disorders (2014): *MEDLINE*. Web. 07 Dec. 2015.

Luger, Thomas A., Thomas E. Scholzen, and Thomas Brzoska. "New Insights into the

    Function of Alpha-MSH and Related Peptides in the Immune System." (2003):

    133-39. Web. 6 Apr. 2016.

Rajora, N., G. Ceriani, and A Catania. "Alpha-MSH Production, Receptors, and

Influence on Neopterin in a Human Monocyte/macrophage Cell Line." Journal of

Leukocyte Biology 59 (1996): 248-53. Print.

Star, R. A., N. Rajora, J. Huang, R. C. Stock, A. Catania, and J.M. Lipton. "Evidence of

Autocrine Modulation of Macrophage Nitric Oxide Synthase by Alpha-

melanocyte-stimulating Hormone. Proceedings of the National Academy Sciences

92.17 (1995): 8016-020. Web.

Tortora, Gerard. J., and Bryan Derrickson. Principles of Anatomy and Physiology.

Hoboken, NJ: John Wiley, 2014. Print.