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The effects of β2 adrenergic receptor activation on macrophage cholesterol accumulation and inflammatory responses
Crystal Wood, Biological Sciences, Dr. Daping Fan, Cell Biology

Abstract:
With the number of cases of cardiovascular diseases, which are most commonly caused by atherosclerosis, continuously rising, it is important to see how different lifestyle factors can contribute to one’s health. If limiting the amount of social stress an individual experiences can cause a decrease in the prevalence of these diseases, it will be important for physicians to encourage patients to avoid possible stressors or determine ways to cope with psychosocial stress in order to decrease their risks for such diseases. In this study, Clenbuterol, a β2 adrenergic agonist, was used to activate the β2 adrenergic receptor on macrophages in order to mimic one way in which macrophages may be affected during an instance of psychosocial stress. The expression of the cholesterol uptake gene CD36 was examined and the cholesterol uptake in aggregated-LDL treated macrophages was measured. Macrophage inflammatory responses to LPS in the presence of Clenbuterol were also examined. It was determined that while CD36 levels increased in macrophages treated with Clenbuterol, no difference in cholesterol levels was observed compared with untreated macrophages. It was also found that a Clenbuterol pretreatment caused increased expression of proinflammatory cytokines such as MCP1 and IL-1β, while cotreatment with Clenbuterol caused the opposite effect (a decrease in these cytokines).

Introduction:
Macrophages are leukocytes that play a major role in the human immune response. A macrophage ingests particles that are foreign to the body and digests them.
Macrophages can quickly change their function in response to local microenvironmental signals (Murray & Wynn, 2011). It has been shown that social stress plays a role in inflammatory responses in the body. Increased levels of social stress are thought to be able to induce inflammatory responses (Scislowska-Czarnecka et al, 2004). Social stress is defined as the physical, mental, and emotional responses to a particular stimulus that results from relationships with others and one’s social environment. For part of my thesis, I will be testing the hypothesis that one component of the psychological response social defeat stress, β2 adrenergic activity, intensifies macrophage inflammatory responses. The proinflammatory cytokines produced can promote the interaction of endothelial cells and leukocytes, which may contribute to the progression of atherosclerosis (Ikonomidis et al, 1999).

Psychosocial stress has been shown to be a risk factor for cardiovascular diseases, especially atherosclerotic coronary artery disease. In previous research, it has been demonstrated that certain psychiatric disorders can promote an unfavorable lipid profile (Chuang et al, 2010). Many such psychiatric disorders can be initiated or worsened by experiencing social stress. Atherosclerosis, the major cause of many cardiovascular diseases including heart attack and stroke, is a chronic inflammatory disease characterized by accumulation of cholesterol and infiltration of macrophages (Singh et al, 2002). Macrophages have specific receptors to recognize normal and modified lipoprotein particles including LDL (low-density lipoprotein) receptors and scavenger receptors (Brock, 2015). Macrophages take up excessive LDL and modified LDL and are not able to efficiently let cholesterol out, which leads to their transformation into foam cells. Foam cells are macrophages loaded with lipids that indicate plaque build up.
Macrophage foam cell formation is the first step of atherogenesis. This accumulation of cholesterol can lead to various health problems associated with cellular toxicity including intracellular cholesterol crystallization, oxysterol formation, and triggering of apoptotic signaling pathways (Tabas, 2002). Adrenergic receptors also play a role in lipid metabolism. It has been reported from previous studies that beta-adrenergic stimulation increases lipolysis (the breakdown of lipids) and inhibits lipoprotein lipase activity, while blockage of these receptors increases plasma triglyceride levels and lowers high density lipoprotein (Smith, 1983). Previous research also shows that β blockers (beta-adrenergic antagonists) have a cardioprotective effect and lower the risk of atherosclerosis (Ulleryd et al, 2014). β2 adrenergic activity could be a significant contributor to the physiological/pathological response to stress because it is a significant component of action of the body’s naturally occurring catecholamines such as epinephrine and norepinephrine.

Literature shows that psychosocial stress can also negatively influence blood pressure, heart rate, and cause inflammation, ultimately contributing to acceleration of atherosclerosis. Inflammatory responses of macrophages to atherogenic stimuli, including bacterial component LPS, further promote atherosclerosis. It has also been concluded that mental stressors can cause rapid elevations in serum cholesterol concentration (Muldoon et al, 1992). It is my hypothesis that activation of the β2 adrenergic receptor by Clenbuterol will increase macrophage cholesterol accumulation by increasing CD36 expression and enhance inflammatory responses to LPS, evidenced by increased MCP1 and IL-1β.
Our preliminary mouse study showed that social defeat stress increased lipid accumulation in peritoneal macrophages in ApoE−/− mice, without an effect on plasma cholesterol and triglyceride concentration. This preliminary study involved subjecting apoE−/− mice to social defeat stress for 10 days followed by either one or two weeks of high fat diet feeding at which point they were sacrificed for analysis. All mice shifted into the “susceptible” category of defeat stress reaction as indicated by their performance in a social avoidance test 24 hours after the 10 days of defeat stress, thus demonstrating that they were significantly affected. This experiment aims to test if increased macrophage cell formation and macrophage inflammatory responses contribute to psychosocial stress accelerated atherosclerosis as the preliminary mouse study suggests. Because macrophages express β2 adrenergic receptor activity at relatively high levels, the experiment has been designed to test the effects of this β2 adrenergic receptor activation on lipid uptake and inflammatory response to LPS.

**Materials and Methods:**

First, we tried treating macrophages with various concentrations of Clenbuterol for different periods of time with little to no effect on cholesterol uptake/efflux associated mRNA and protein expression. We then decided to examine if a double treatment of Clenbuterol would be more effective.

*Transcriptional Effects of Clenbuterol Treatment on Macrophage Cholesterol Homeostasis Genes:*

In order to test the effects of the β2 agonist Clenbuterol on the expression of genes related to cholesterol homeostasis, we performed the following:
Three milliliters of Thioglycollate was injected intraperitoneally into one male C57BL/6 mouse. Three days later, the mouse was sacrificed using Isoflurane and cervical dislocation. Peritoneal macrophages were obtained by 2x wash with 10ml of cold, sterile PBS. Macrophages were centrifuged for five minutes at 1400rpm, and the supernatant was vacuumed away. The cells were then resuspended in 1ml of DMEM supplemented with 10% FBS and penicillin/streptomycin (P/S). Cells were counted, plated in a six-well plate with three million cells per well, and incubated at 37°C for 2 hours. The cells were washed 2x with 2ml of PBS and 2ml serum-free DMEM+P/S was added to each well. The cells were then incubated at 37°C overnight. Next, the experimental dilution of Clenbuterol (1.35x10⁻⁷M) was made to model the effects of psychosocial stress on macrophages. This was done by diluting 0.5µl of 1x10⁻¹M with 499.5µl of DMEM+P/S, yielding 500µl of 1x10⁻⁴M of drug, and adding 8.25µl of this to 5991.75µl DMEM+P/S to yield a final volume of 6ml 1.35x10⁻⁷M Clenbuterol. Macrophages were then treated with 2ml of the drug and put in the incubator at 37°C for 8 hours. After 8 hours, the drug media was vacuumed away, replaced, and the cells were incubated for 8 more hours. After the second 8 hour interval, the media was vacuumed away and the cells were rinsed with 2ml PBS. Afterwards, 700µl of Quiazol was added to lyse the cells. The cell lysate was collected in 1.5ml Eppendorf tubes and stored at -80°C. The next day, total RNA extraction was performed on samples using Quagen RNA Isolation kit. The RNA was then diluted 1:20 and quantified. Reverse transcription was performed using BioRad cDNA synthesis kit. qPCR was then performed on samples for CD36 genes using 18S as the internal control. Statistical analysis was performed and used to create graphs in GraphPad Prism 5. The same experiment was performed again and a 16 hour continuous
Clenbuterol treatment group was added to be sure that it was not simply the duration of the treatment, but the washout after 8 hours that was causing the response.

*Effect of Clenbuterol Treatment on Macrophage Cholesterol Uptake:*

In order to test the effects of the β2 agonist Clenbuterol on cholesterol uptake we performed the following:

Three milliliters of Thioglycollate was injected intraperitoneally into two male C57BL/6 mice. Three days later, the mice were sacrificed using Isoflurane and cervical dislocation. Peritoneal macrophages were obtained by 2x wash with 10ml of cold, sterile PBS for each mouse. Macrophages were centrifuged for five minutes at 1400 rpm, and the supernatant was vacuumed away. The cells were then resuspended in 1ml of DMEM supplemented with 10% FBS and P/S. Cells were counted, plated in a six-well plate (three million cells per well) with three samples labeled “LDL only” (not treated with Clenbuterol) and three samples labeled “LDL+β2 Treatment”. The plates were incubated at 37°C for 2 hours. The cells were washed 2x with 2ml of PBS and 2ml serum-free DMEM+P/S was added to each well. The cells were incubated at 37°C overnight. Next, a double dose of the experimental dilution of Clenbuterol (2.70x10⁻⁷M) was made to model the effects of psychosocial stress on macrophages. This was done by diluting 0.5μl of 1x10⁻¹M with 499.5μl of DMEM+P/S, yielding 500μl of 1x10⁻⁴M of drug, and adding 16.5μl of this to 5983.5μl of DMEM+P/S to yield a final volume of 6ml 2.70x10⁻⁷M Clenbuterol. After 24 hours of incubation, 120μl of LDL (50μg/ml) was vortexed for one minute and 60μl of the LDL was added to 12ml DMEM+PS, while the other 60μl was added to the Clenbuterol experimental treatment medium. Two milliliters of the
DMEM+PS with LDL was added to each of the three wells labeled “LDL” and two milliliters of the Clenbuterol treatment medium was added to each of the “LDL+β2 Treatment” wells. After 48 hours, the media was vacuumed away and the wells were rinsed 2x with PBS. The plates were put under the hood with the lid slightly open to allow the wells to dry. Once the plates were completely dry, 500μl of isopropanol was added to each well and left in the hood overnight. The isopropanol was collected and put in labeled Eppendorf tubes. These tubes were left under the hood for two days to allow the Isopropanol to evaporate. In the wells, 500μl of 0.1M NaOH was added and incubated at room temperature for 15 minutes. The cells were then transferred to new labeled Eppendorf tubes. The Lowry Method was performed on these samples to determine protein concentration as an internal control for cell number. Once the Isopropanol evaporated from the tubes that were placed under the hood, 200μl of Cholesterol Assay Buffer was added and the samples were diluted 1:5. Cholesterol quantification was performed using the Cellular Cholesterol Quantification Kit (Sigma-Aldrich).

Clenbuterol’s Effect on Macrophage Response to an Inflammatory Stimulus:

In order to test the impact of the β2 agonist Clenbuterol on macrophage inflammatory response, we performed the following:

Three milliliters of Thioglycollate was injected intraperitoneally into three male C57BL/6 mice. Three days later, the mice were sacrificed using Isoflurane and cervical dislocation. Peritoneal macrophages were obtained by 2x wash with 10 of cold, sterile PBS. Macrophages were centrifuged for five minutes at 1400 rpm, and the supernatant was
vacuumed away. The cells were then resuspended in 1ml of DMEM supplemented with 10% FBS and P/S. Cells were counted, plated in two twelve-well plates with two million cells per well (Pretreatment & Concomitant Treatment), and incubated at 37°C for 1 hour. The cells were washed 2x with 2ml of PBS and 2ml serum-free DMEM was added to each well. The cells were then incubated at 37°C overnight. The plates were treated with 2ml of the appropriate medium as follows:

**Pretreatment**

8AM: Started Clenbuterol Pretreatment.

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2PM: Started LPS treatment (all in DMEM+PS)

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8PM: Stopped Pretreatment Experiment. Treatment was removed. The wells were washed with 2ml PBS 2x and 1ml of Quiazol was added to lyse the cells. The cell lysate was collected in Eppendorf tubes labeled 1-12 and stored at -80°C.

**Concomitant**

10AM: Started Concomitant Treatment.

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DMEM (Vehicle) | Cle (135 nM) | LPS (50ng/ml) | Cle(135nM)+LPS(50ng/ml)

4PM: Stopped Concomitant Treatment Experiment. Treatment was removed. The wells were washed with 2ml PBS 2x and 1ml of Quiazol was added to lyse the cells. The cell lysate was collected in Eppendorf tubes labeled 13-24 and stored at -80°C. Total RNA extraction, reverse transcription, and qPCR was performed for all 24 samples.

Results:

*Transcriptional Effects of Clenbuterol Treatment on Macrophage Cholesterol Homeostasis Genes:*

It was determined that under 8h+8h conditions, Clenbuterol upregulates CD36. Under a continuous 16 hour Clenbuterol treatment, no change in CD36 expression was evident.

![CD36 expression graph](image)

Figure 1 Under a double 8-hour treatment, Clenbuterol upregulates CD36 expression.
Figure 2 The second double 8-hour Clenbuterol treatment had similar results. There was no significant difference with the continuous 16-hour treatment.

It is important to note that Clenbuterol double treatment paradoxically lowered cholesterol accumulation in the macrophages.

*Effect of Clenbuterol Treatment on Macrophage Cholesterol Uptake:*

There was no significant difference in the intracellular concentrations of total cholesterol, free cholesterol, or cholesterol esters in the macrophages treated with only LDL versus the macrophages treated with LDL + Clenbuterol.
Figure 3 There was no significant difference in total cholesterol levels between the three samples treated with Clenbuterol and the three samples that did not receive the Clenbuterol treatment.

Figure 4 There was no significant difference in free cholesterol levels between the three samples treated with Clenbuterol and the three samples that did not receive the Clenbuterol treatment.
Figure 5 There was no significant difference cholesterol ester levels between the three samples treated with Clenbuterol and the three samples that did not receive the Clenbuterol treatment.

Clenbuterol’s Effect on Macrophage Response to an Inflammatory Stimulus:

It was shown that for the Pre-treatment plate, Clenbuterol increased MCP1 expression in macrophages in response to LPS stimulation. Due to p-values greater than 0.05, no other conclusions can be drawn from the data, but trends are still observable. The pretreatment had no observable effect on CD36 expression. The Clenbuterol pretreatment increased IL-1β expression in macrophages in response to LPS stimulation.
Figure 6 Pretreatment with Clenbuterol caused no significant change in levels of CD36 expression in macrophages in response to LPS stimulation.

Figure 7 Pretreatment with Clenbuterol caused increased levels of MCP1 expression in macrophages in response to LPS stimulation.
Figure 8 Pretreatment with Clenbuterol caused no significant change in levels of IL1β expression in macrophages in response to LPS stimulation.

Discussion:

The results from the double Clenbuterol treatment suggest that macrophage treatment with Clenbuterol, a β2 adrenergic receptor agonist, can upregulate CD36 expression, but only under specific multiple treatment conditions. CD36 is a scavenger receptor protein involved in lipid uptake. LDL may be taken up by scavenger receptors such as CD36 after being oxidized, leading to the accumulation of LDL cholesterol and foam cell formation (Podrez et al, 2000). Foam cell formation often leads to the buildup of atherosclerotic plaques and is the first stage in the development of atherosclerosis. Foam cells build up to create an atheromatous core and eventually these plaques become necrotic, made up of lipids, cholesterol crystals, and cell debris (Bobryshev, 2005). Because Clenbuterol models some of the effects of psychosocial stress on macrophages, this data helps to determine one of the mechanisms by which psychosocial stress could
accelerate the accumulation of cholesterol and exacerbate inflammation, ultimately leading to increased risk of atherosclerosis. However, it is important to note that under none of the single Clenbuterol treatment conditions did any of the cholesterol homeostasis genes analyzed (CD36, ABCA1, LXRα, SRA-1) demonstrate any transcriptional changes. It is also important to note that this increase in the scavenger receptor transcription was not accompanied by an increase in cholesterol accumulation, but instead a decrease in macrophage cholesterol was observed after the 8h+8h double treatment with Clenbuterol. This could be due to the fact that increased mRNA expression does not always lead to increased functional protein expression, or a more significant effect brought about by Clenbuterol on a competing pathway.

Previous research also indicates that the proinflammatory cytokine IL-1β enhances cholesterol uptake from human macrophages by upregulating oxidized LDL receptors, which can often lead to foam-cell formation; IL-1β mRNA and protein have even been isolated from human atherosclerotic lesions (Ikonomidis et al, 1999). While the results from the Clenbuterol + LPS Pretreatment study reflect these same findings, the results were not quite statistically significant. However, the results did support the idea that through activation of the β2 adrenergic receptor, Clenbuterol increases expression of MCP-1 transcription in macrophages in response to LPS stimulation. Monocyte chemoattractant protein-1 (MCP-1) functions in recruiting monocytes and macrophages to the subendothelial cell layer, and deposits of lipids within these cells lead to the formation of atherosclerotic lesions (Harrington, 2008). Therefore, β2 adrenergic stimulation in macrophages could increase the risk of developing atherosclerotic lesions.
It can be determined from the data that treatment with Clenbuterol causes an increase in CD36 and MCP-1 mRNA expression. This data may also suggest that β2 activation does not increase cholesterol accumulation in macrophages. These results do not support the original hypothesis that β2 activation increases cholesterol accumulation. This could be due to a variety of reasons, including experimental errors involving methods used in cell culture or pipetting. This finding could also be a result of an experimental design error. If CD36 expression was increased by Clenbuterol, cholesterol uptake may be increased; however, cholesterol efflux may also increase, thus the net accumulation would not change. Another consideration would be that during the 48 hour cholesterol loading period, CD36 expression returned to normal levels so quickly that uptake was not actually increased.

For future studies, different time points should be examined during the cholesterol loading period in order to eliminate one possible source of experimental design error. Cholesterol efflux rates should also be examined in macrophages treated with Clenbuterol. Macrophages could possibly also be treated with a selective β2 adrenergic antagonist such as ICI118551 to determine if opposite trends are observed. A continuous study of this topic is important in order to identify some of the possible risk factors for coronary atherosclerosis and determine possible mechanisms for avoidance of these cardiovascular diseases. This is the first study that directly tests if activation of the β2 adrenergic receptor can enhance macrophage foam cell formation and determines some of the possible underlying mechanisms.
References:


