On the inflammatory response of skeletal muscles to a high-lipid diet

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Abstract

**Background:** The low-grade, chronic inflammation is a process characterized by slightly elevated levels of pro-inflammatory molecules. It can be induced by increased levels of saturated fatty acids which could affect toll-like receptor signaling pathways. In this experiment we estimated the degree of low-grade inflammation in different muscles of Wistar rats subjected to a high-lipid diet.

**Methods:** To study the effect of the high-fat diet on the development of inflammation in skeletal muscles we used sixty, 8-week-old male Wistar rats. The animals were subjected to standardized rodent food – a high-fat and a standard diet. After the period of time needed to provoke inflammation we changed the diet in order to collect data for the duration of the process. We used the quantitative ELISA method to determine the degree of the tissue inflammatory process, studying the levels of pro- and anti-inflammatory marker molecules.

**Results:** We established that the high-fat diet changes the levels of the measured proinflammatory molecules to an increase. Their levels in the studied skeletal muscles were slightly elevated compared to the controls. We detected that the inflammatory process depends on time as well. A longer period of time leads to an additional increase in the levels of the cytokines in some muscles, while their levels remain almost the same in others. The replacement of the high-lipid diet with a standard one causes the muscles to restore of their normal, non-inflammatory phenotype. At the same time, the levels of the anti-inflammatory molecule show almost no change during the experiment.

**Conclusions:** Skeletal muscles develop inflammation as a result of the intake of a high-lipid diet. The process is low-grade, chronic and its duration is as long as the high-fat food persists in the diet of the animals. The insignificantly changed levels of the anti-inflammatory molecule indicate that the muscle tissue does not, or barely exerts control over the development of this process.

**Background**

Inflammation is a normal component of the human self-defense mechanisms [1]. It is a cellular, a specifically organismal response to various stressful situations such as viral infections, tissue damage, etc. Regardless the reason that provokes it, it is considered to be a process that aids the restoration of the functional and phenotypic homeostasis of the damaged cell, tissue or organ [2].

It has been reported that under specific conditions such as obesity, some tissues develop active inflammatory process, even in the absence of infection or any visible damage. Such a process is atypical, low-grade and chronic. This type of inflammation differs from the acute inflammation since the levels of the circulating pro-inflammatory marker molecules are only slightly elevated [3, 4]. It appears to be caused by many different factors such as excessive calories intake, changed food composition and unusual homeostasis [4].
The prolonged consumption of a high-fat diet leads to abnormal growth of the adipose tissue and development of obesity. In turn, obesity has been associated with changes in the immune cells composition in the tissue and development of low-grade, chronic inflammation [Johnson AR et al, 2012] [5]. Many authors have published that the liver also develops low-grade inflammation as a result of obesity and a changed number of macrophages in the organ [6, 7, 8]. At the same time clear and reliable information about other metabolically active tissues is still insignificant. The aim of the present study was to evaluate the development of dietary-induced inflammation in skeletal muscles by measuring the levels of the proinflammatory, acute-phase proteins – C-reactive protein (CRP) and serum amyloid-A (SAA), and the anti-inflammatory interleukin-4 (IL4).

**Methods**

This study has an approval for the usage of laboratory animals in experiments from the Bulgarian Agency for Food Safety (BAFS – resolution №55/23.06.2016), and it is in accordance with the ethical standards of the Medical University of Plovdiv (resolution of the University Ethics Committee – №P-1041/25.04.2017).

Sixty, 8-week-old male *Wistar* rats (weight 130–180 g) were used for the needs of this experiment. They were randomly divided into two groups:

1. Control group (C, n = 24), fed with standard rodent food (*D12450H, Research Diets, Inc.*) for 14 weeks.
2. Experimental group (E, n = 36), fed with a high-fat diet (*D12451 – Research Diets, Inc.*) for 14 weeks.

All animals were maintained under standard housing conditions, they were all fed *ad libitum* and were housed at 12 h light/dark cycles [9]. Twelve animals were taken at random from each group after 14 weeks and tissue samples were collected and stored [9]. The tissue samples obtained were from three different skeletal muscles – *musculus gastrocnemius* (*m. Gas*), *musculus soleus* (*m. Sol*) and *musculus extensor digitorum longus* (*m. EDL*). The decision to collect these muscles was based on the specific distribution of muscle fibers with characteristic physiology and metabolism – *m. Sol* consists of 85% type I muscle fibers, *m. EDL* – 96% type II muscle fibers and *m. Gas* – 51% type I and 49% type II muscle fibers [Delp MD and Duan C.] [10].

The remaining animals were divided into three new groups as follows:

1. The animals from the control group were established into a new control group (CC, n = 12). They all continued to be fed with standard food (*D12450H, Research Diets, Inc.*) for four more weeks.

The experimental (E) group was split into two new groups:

1. Experimental group (EE, n = 12), fed the same high-fat diet for four more weeks
2. Experimental group (EC, n = 12) – the diet of the animals from this group was changed from the high-fat to the standard diet. They were all fed with the standard food for the next four weeks.
The duration period of the experiment was eighteen weeks in total for the animals from the three new groups. The same procedure was followed when the tissue samples were collected at the end of our experiment [9].

The quantitative ELISA method was used for the concentration of CRP, SAA and IL-4 in the tissue homogenates to be determined, while the Lawry method [Lawry et al, 1951] [11] was used for the determination of the total protein concentration of the same samples. The levels of CRP, SAA and IL-4 in the studied tissues are present as a ratio of the concentration of the marker in the sample to the total protein concentration in the same tissue sample (ng CRP/mg Protein, ng SAA/mg Protein and pg IL-4/mg Protein).

The collected data was statistically processed by SPSS, v.19.0 (SPSS Inc., Chicago, IL, USA). The results were compared by Kruskal-Wallis test for non-parametric data and are present as median (25th – 75th percentile). Differences with p-values less than 0.05 were considered as statistically significant. The Dunn's post-hoc test was performed for those groups where statistical difference was found.

Results

1. Development of inflammation in m. Gas

The levels of both proinflammatory marker molecules CRP and SAA, measured in samples from m. Gas, showed statistically significant differences among the experimental groups. The levels of CRP, measured in the experiment group “E” – 15.07 ng/mg (8.39–20.19 ng/mg) – were significantly higher than the CRP levels in the control group “C” – 5.61 ng/mg (4.02–7.84 ng/mg), p < 0.05. This difference was even more pronounced when the experiment group “EE” – 26.93 ng/mg (17.6–38.77 ng/mg) was compared with either of the control groups “C”, p < 0.01, or “CC” – 5.32 ng/mg (2.91–6.99 ng/mg), p < 0.01. Differences were detected even between groups “E” and “EE”, p < 0.05. The detected values for CRP in the group “EC” – 15.04 ng/mg (11.61–20.62 ng/mg) were still high. They were close to those detected for groups “E” and “EE”, p > 0.05 and much higher than those observed in the control groups, p < 0.05 (Fig. 1A).

The changes in the levels of SAA followed a similar pattern as the changes for CRP. The levels were high and there was a statistically significant difference detected on week 14. The levels in the control group “C” – 26.12 ng/mg (22.61–38.64 ng/mg) were much lower than in the experimental group “E” – 64.58 ng/mg (39.18–88.76), p < 0.01. In contrast to the changes in the levels of CRP, the SAA levels did not show any dependence on time. The levels of SAA in the experimental group “EE” – 45.75 ng/mg (35.39–70.78 ng/mg) – were almost the same as its quantity in the group “E”, p > 0.05, but significantly higher than in both control groups “C” and “CC” – 24.71 ng/mg (22.61–31.39 ng/mg), p < 0.05. The quantitative analysis of SAA in the group where the diet was changed from high-fat to standard showed that the marker levels are of intermediate values. They were close to the values of the control groups “C” and “CC” on one side, p > 0.05, but comparatively lower than those of the experiment groups “E” and “EE”, p < 0.05, on the other (Fig. 1B).
The levels of the anti-inflammatory IL-4 molecule were very close and with no significant differences among the groups: "C": 7.74 pg/mg (3.74–15.32 ng/mg), "CC": 5.62 pg/mg (4.29–7.55 ng/mg), "E": 11.73 pg/mg (9.19–14.7 ng/mg), "EE": 9.97 pg/mg (7.35–17.47 ng/mg) и "EC": 9.52 pg/mg (4.19–12.24 ng/mg), p > 0.05 (Fig. 1C).

2. **m. Sol** also develops dietary-induced inflammation

We established that *m. Sol* responds to the high-fat diet with changes in the levels of both proinflammatory markers, p < 0.01. The levels of CRP were high in both groups fed only with the high-fat diet. The quantity of CRP in the group "E" – 13.82 ng/mg (7.2–14.57 ng/mg) and "EE" – 13.1 ng/mg (11.61–16 ng/mg) was significantly higher when compared with the control group “C” – 6.04 ng/mg (3.21–9.44 ng/mg), p < 0.05. The CRP levels measured in the second control group “CC” – 8.34 ng/mg (7.34–9.55 ng/mg) and the “EC” group – 11.27 ng/mg (6–14.79 ng/mg) were similar to those of the control group “C”, p > 0.05, and the groups “E” and “EE”, p > 0.05 (Fig. 2A).

The quantitative analysis of SAA in samples from *m. Sol* showed no significant differences between the control groups “C” – 25.63 ng/mg (20.31–33.19 ng/mg), and “CC” – 27.27 ng/mg (17.39–47.6 ng/mg) with the experiment group “E” – 27.02 ng/mg (19.48–30.02), p > 0.05. However, the prolonged consumption of the lipid-rich food causes changes in the levels of the proinflammatory marker – the levels were significantly higher in the group “EE” – 52.54 ng/mg (29.46–79.5 ng/mg). The quantity of SAA in this group was much higher than in all other groups, p < 0.05. We also detected that when the animals stopped taking the high-lipid food and went back to the standard diet, the values of the marker – group “EC” – 27.93 ng/mg (22.44–36.4 ng/mg) were also decreased and close to their normal, control group values, p > 0.05 (Fig. 2B).

The quantitative analysis of the IL-4 levels in *m.Sol* demonstrated a lack of statistically significant differences amongst the compared groups: "C" – 37.18 pg/mg (26.75–64.15), "CC" – 36.45 pg/mg (26.75–46.12), "E" – 44.12 pg/mg (28.72–56.98), "EE" – 45.94 pg/mg (35.35–58.23) and "EC" – 27.55 pg/mg (17.59–33.41), p > 0.05 (Fig. 2C).

3. Dietary-induced changes in **m. EDL**: 

Development of inflammation was detected in *m. EDL*, similarly to the previously described muscles. Using the Kruskal-Wallis test, we established an increase in the levels of CRP only. p < 0.01. The comparison between the values in the group “C” – 9.1 ng/mg (2.4–11.58 ng/mg) on one hand, and the groups “E” – 15.76 ng/mg (9.83–24.9 ng/mg) and "EE" – 13.76 ng/mg (12.82–28.14 ng/mg), p < 0.05, on the other, gave evidence for us to state that there is an inflammatory process. Similar dependence was observed when both the “E” and “EE” groups were compared to the control group “CC” – 6.39 ng/mg (3.01–7.77 ng/mg), p < 0.01. Decreased CRP levels as a result of suspended high-lipid intake were detected in this muscle only. The CRP values in the “EC” – 9.6 ng/mg (7.5–12.16 ng/mg) are much lower than those in the experimental groups, p < 0.05 and very close to the levels in the control groups, p > 0.05 (Fig. 3A).
The comparison of the levels of SAA in tissue homogenates from m. EDL: "C" – 12.73 ng/mg (10.1–16.85 ng/mg), "CC" – 13.28 ng/mg (12.61–14.95 ng/mg), "E" – 16.81 ng/mg (13.83–18.74 ng/mg), "EE" – 22.77 ng/mg (12.47–30.4 ng/mg) и "EC" – 14.52 ng/mg (13.28–17.2 ng/mg), did not show any significant differences, p > 0.05. (Fig. 3B).

It was interesting to find that the changes in the quantity of IL-4 did not follow the pattern, detected in the previously described muscles. The quantity of IL-4 was significantly higher in the “EE” group – 21.81 pg/mg (14.41–24.73 ng/mg), than in the control groups “C” – 6.61 pg/mg (4.37–11.65 ng/mg) and “CC” – 4.84 pg/mg (4.04–6.05 ng/mg), p < 0.01. At the same time the amount of IL-4 established in the group “EC” – 4.01 pg/mg (3.53–14.17 ng/mg) was almost the same as the amount of the marker in the groups “C” and “CC”, p > 0.05. (Fig. 3C)

**Discussion**

The process of tissue inflammation requires well-coordinated cytokine secretion, as well as activation and infiltration of immune cells in the tissues [12]. Our experiment established increased levels of both proinflammatory marker molecules in the tested tissues. The elevation of their levels follows a different pattern and depends on time in a different way for each of the markers. At the same time, the changes in the levels of the anti-inflammatory molecule follow neither the same pattern, nor similar dependence on time. All the mentioned above could be a result of an impaired intercellular communication, as well as disturbance in the normal differentiation of the cells and/or normal tissue remodeling [13].

Many studies have demonstrated that both pro- and anti-inflammatory molecules are present in the food [14, 15]. It has been reported that saturated fatty acids can influence the development of an inflammatory process, affecting intracellular signaling pathways that use nuclear factor kappa-B and the family of peroxisome proliferator-activated receptors [16]. The proinflammatory effects are triggered by a family of receptors called toll-like receptors (TLR), that are found on the plasma membrane of the immune system cells and on muscle cells [17]. It has also been reported that the number of these receptors on the muscle cells vary and depends on the diet [18].

It has been proposed that the muscle tissue develops inflammation in obesity. This type of inflammation depends on the increased infiltration of immune cells on one side, and the proinflammatory phenotype of the adipose tissue located in the skeletal muscles, on the other. The inflammatory process and muscle metabolism could be also affected by an increased influx of free fatty acids (FFA) and/or proinflammatory molecules, secreted by many other cells and tissues, including the visceral adipose tissue [19].

Another possible reason for the development of an inflammatory process could be the tissue composition. Tissue macrophages are constantly present in the skeletal muscles, as well as in other tissues like the adipose tissue and liver. They are responsible for the tissue damage repair [20]. Additional infiltration of a tissue by activated macrophages could be due to an increased concentration of FFA, tissue damages or adipose tissue depots in skeletal muscles [21, 22]. Tissue macrophages could be also...
transformed into activated macrophages that exhibit proinflammatory phenotype. All the mentioned above state that the prolonged consumption of a high-lipid diet could provoke the development of inflammation in the skeletal muscles [23].

Our experiment detected that the levels of CRP increase faster than the levels of SAA or that there is a complete lack of changes in the levels of SAA. The changes in the CRP levels were dependent on time only in \textit{m.Gas}. This could be explained with the assumption that the high-lipid diet could affect the amount of the proinflammatory molecules at many levels. \textit{Firstly}, the increased levels of FFA, are connected with an increased infiltration of macrophages into the muscles. These macrophages are found mainly in intramuscular fat depots, which increase in number and volume in obesity [24]. It has been proposed that proinflammatory molecules could be released from such depots to provoke paracrine effects on the development of inflammation in the neighboring muscle cells [25, 26]. \textit{Secondly}, it has been reported that muscle infiltrated macrophages secrete proinflammatory cytokines such as IL-6 when placed in a medium with excess of FFA [27]. \textit{Thirdly}, in vitro studies with \textit{m. Sol} placed in a FFA-rich medium, have demonstrated the release of proinflammatory molecules such as IL-6 and TNF-α from the muscle cells [28, 23]. All these observations propose the existence of many and complex interrelationships between the cells to respond to the intake of the high-lipid diet [Pillon et al, 2012] [23]. All the reasons listed above might be responsible for the different timing of the inflammatory process as well as for modulation of its development.

The receptors that belong to the group of the TLRs are among the most important participants in the development of inflammation and exhibition of the effects of a high-lipid diet. It has been reported that the expression of TLR2 and TLR4 is higher in skeletal muscles subjected to a high-lipid diet [18]. Overexpression of the receptor, higher body weight, decreased glucose and insulin tolerance were reported for rodents who consumed such diet [29].

Studies with human patients with obesity and diabetes mellitus type II have demonstrated increased levels of macrophages and T-cells in the skeletal muscles. Increased macrophage levels have also been observed in the skeletal muscles of healthy patients as a result of the intake of a high-lipid diet even for a short period of time [30].

All the reasons mentioned above link the consumption of a high-fat diet with the development of inflammation in the skeletal muscles. The inflammatory process is characterized with increased levels of proinflammatory molecules which in turn mediate the development of inflammation in the muscle tissue [18].

**Conclusions**

Skeletal muscles constantly respond to the changes in their surrounding environment. The increased levels of the proinflammatory molecules observed in our \textit{in vivo} experiment, support the hypothesis that skeletal muscles develop inflammation as a result of the intake of a high-lipid diet. This inflammation affects all the muscles tested during the experiment. The insignificantly changed levels of the anti-
inflammatory molecule indicate that the muscle tissue does not, or barely exerts control over the 
development of this process. The tendency for the levels of the proinflammatory markers to decrease 
after the high-fat diet was suspended gives us a reason to state that the dietary-induced inflammation is 
a reversible process.

**Abbreviations**

CRP: C-reactive protein

SAA: Serum amyloid-A

IL-4: Interleukin-4

IL6: Interleukin-6

m. Gas: Musculus gastrocnemius

m. Sol: Musculus soleus

m. EDL: Musculus extensor digitorum longus

FFA: Free fatty acids

TNF-α: Tumor-necrosis factor-α

TLR4 and TLR6: Toll-like receptors 4 and 6

**Declarations**

*Ethics approval and consent to participate*

This study has an approval for the usage of laboratory animals in experiments from the Bulgarian 
Agency for Food Safety (BAFS – resolution №55/23.06.2016), and it is in accordance with the ethical 
standards of the Medical University of Plovdiv (resolution of the University Ethics Committee – №P- 

*Availability of data and materials*

The datasets used and/or analyzed during the current study are available from the corresponding author 
on reasonable request.

*Competing interests*

None.
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**Authors contribution**

All authors distributed equally to the submission of a scientific project for consideration, the data from which gave rise to this manuscript. The main idea and the initial organization belonged to associate professor Vassil Kamenov, MD who unfortunately passed away in early 2021. I am not sure whether to add his name to the authors of this manuscript, so I am presenting his contribution here.

The team of researchers from the departments of Medical Biochemistry and Medical Biology, Iliyan Dimitrov, Dr. A. Bivolarska, T. Stankova, I. Dimov and M. Draganova-Filipova performed the extraction and purification of protein-rich samples from isolated muscles. They performed the ELISA method to obtain the data and also did the statistical analyzes.

Professor Katerina Georgieva and her colleagues from the Department of Physiology, prof. N. Boyadjiev, Dr. P. Angelova, and Dr. D. Popov developed and realized the high-lipid model, including the preparation and procedures for all the documents needed.

Associate professor Slavi Delchev from the Department of Human Anatomy and his colleagues Dr. E. Daskalova and Dr. F. Gerginska collected the muscles needed for this experiment. They also prepared the samples in order to be stored for future experiments.

All authors were involved equally in preparing the manuscript, providing information for the distinct paragraphs and chapters.

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**References**


Figures
Figure 1

Changes in the levels of CRP, SAA and IL-4 in tissue homogenates, obtained from \textit{m. gas}. 
Figure 2

Changes in the levels of CRP, SAA and IL-4 in tissue homogenates obtained from *m. sol.*
Figure 3

Changes in the levels of CRP, SAA and IL-4 in tissue homogenates obtained from *m. EDL*.